

## **Methods And Means To Modulate Programmed Cell Death In Eukaryotic Cells.**

### **Field of the invention**

The invention relates to the use of poly (ADP-ribose) polymerase (PARP) proteins, particularly mutant PARP proteins or parts thereof, and genes encoding the same, to produce eukaryotic cells and organisms, particularly plant cells and plants, with modified programmed cell death. Eukaryotic cells and organism, particularly plant cells and plants, are provided wherein either in at least part of the cells, preferably selected cells, the programmed cell death (PCD) is provoked, or wherein, on the contrary, PCD of the cells or of at least part of the cells in an organism is inhibited, by modulation of the level or activity of PARP proteins in those cells. The invention also relates to eukaryotic cells and organisms, particularly plant cells and plants, expressing such genes.

### **Description of related art**

Programmed cell death (PCD) is a physiological cell death process involved in the elimination of selected cells both in animals and in plants during developmental processes or in response to environmental cues (for a review see Ellis *et al.* 1991; Pennell and Lamb, 1997). The disassembly of cells undergoing PCD is morphologically accompanied by condensation, shrinkage and fragmentation of the cytoplasm and nucleus, often into small sealed packets (Cohen 1993, Wang *et al.* 1996). Biochemically, PCD is characterized by fragmentation of the nuclear DNA into generally about 50 kb fragments representing oligonucleosomes, as well as the induction of cysteine proteinases and endonucleases. The fragmentation of the DNA can be detected by terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) of DNA 3'-OH groups in sections of cells. (Gavrieli *et al.* 1992). Cell death by PCD is clearly distinct from cell death by necrosis, the latter involving cell swelling, lysis and leakage of the cell contents.

In animals, PCD is involved in the elimination or death of unwanted cells such as tadpole tail cells at metamorphosis, cells between developing digits in vertebrates,

overproduced vertebrate neurons, cells during cell specialization such as keratocytes etc. Damaged cells, which are no longer able to function properly, can also be eliminated by PCD, preventing them from multiplying and/or spreading. PCD, or the lack thereof, has also been involved in a number of pathological conditions in humans (AIDS, Alzheimer's disease, Huntington's disease, Lou Gehring's disease, cancers).

In plants, PCD has been demonstrated or is believed to be involved in a number of developmental processes such as e.g., removal of the suspensor cells during the development of an embryo, the elimination of aleurone cells after germination of monocotyledonous seeds; the elimination of the root cap cells after seed germination and seedling growth; cell death during cell specialization as seen in development of xylem tracheary element or trichomes, or floral organ aborting in unisexual flowers. Also the formation of aerenchyma in roots under hypoxic conditions and the formation of leaf lobes or perforations in some plants seem to involve PCD. Large scale cell death in plants occurs during senescence of leaves or other organs. The hypersensitive response in plants, in other words the rapid cell death occurring at the site of entry of an avirulent pathogen leading to a restricted lesion, is another example of PCD in response to an environmental cue.

Animal or plant cells dying in suspension cultures, particularly in low-density cell suspension cultures, also demonstrate the characteristics of PCD.

An enzyme which has been implied to be involved in PCD or apoptosis is poly(ADP-ribose) polymerase. Poly(ADP-ribose) polymerase (PARP), also known as poly(ADP-ribose) transferase (ADPRT) (EC 2.4.2.30), is a nuclear enzyme found in most eukaryotes, including vertebrates, arthropods, molluscs, slime moulds, dinoflagellates, fungi and other low eukaryotes with the exception of yeast. The enzymatic activity has also been demonstrated in a number of plants (Payne *et al.*, 1976; Willmitzer and Wagner, 1982; Chen *et al.*, 1994; O'Farrell, 1995).

PARP catalyzes the transfer of an ADP-ribose moiety derived from NAD<sup>+</sup>, mainly to the carboxyl group of a glutamic acid residue in the target protein, and subsequent ADP-ribose polymerization. The major target protein is PARP itself, but also histones,

high mobility group chromosomal proteins, a topoisomerase, endonucleases and DNA polymerases have been shown to be subject to this modification.

The PARP protein from animals is a nuclear protein of 113-120 kDa, abundant in most cell types, that consist of three major functional domains: an amino-terminal DNA-binding domain containing two Zn-finger domains, a carboxy-terminal catalytic domain, and an internal domain which is automodified (de Murcia and Ménissier de Murcia, 1994; Kameshita *et al.*, 1984; Lindahl *et al.*, 1995). The enzymatic activity *in vitro* is greatly increased upon binding to single-strand breaks in DNA. The *in vivo* activity is induced by conditions that eventually result in DNA breaks (Alvarez-Gonzalez and Althaus, 1989; Ikejima *et al.*, 1990). Automodification of the central domain apparently serves as a negative feedback regulation of PARP.

PARP activity in plant cells was first demonstrated by examining the incorporation of  $^3\text{H}$  from labelled  $\text{NAD}^+$  into the nuclei of root tip cells (Payne *et al.*, 1976; Willmitzer and Wagner, 1982). The enzymatic activity was also partially purified from maize seedlings and found to be associated with a protein of an apparent molecular mass of 113 kDa, suggesting that the plant PARP might be similar to the enzyme from animals (Chen *et al.*, 1994; O'Farrell, 1995).

cDNAs corresponding to PARP proteins have isolated from several species including mammals, chicken, *Xenopus*, insects and *Caenorhabditis elegans*.

Chen *et al.* (1994) have reported PARP activity in maize nuclei and associated this enzymatic activity with the presence of an approximately 114 kDa protein present in an extract of maize nuclei. O' Farrel (1995) reported that RT-PCR-amplification on RNA isolated from maize (using degenerate primers based on the most highly conserved sequences) resulted in a 300 bp fragment, showing 60% identity at the amino acid level with the human PARP protein. Lepiniec *et al.* (1995) have isolated and cloned a full length cDNA from *Arabidopsis thaliana* encoding a 72 kDa protein with high similarity to the catalytic domain of vertebrate PARP. The N-terminal domain of the protein does not reveal any sequence similarity with the corresponding domain of PARP from vertebrates but is composed of four stretches of amino acids (named A1, A2, B and C) showing similarity to the N-terminus of a number of nuclear and

DNA binding proteins. The predicted secondary structure of A1 and A2 was a helix-loop-helix structure.

The Genbank database contains the sequences of two cDNAs from *Zea mays* for which the amino acid sequence of the translation products has either homology to the conventional PARP proteins (AJ222589) or to the non-conventional PARP proteins, as identified in *Arabidopsis* (AJ222588)

The function(s) of PARP and poly-ADP ribosylation in eukaryotic cells is (are) not completely clear. PARP is involved or believed to be involved either directly or indirectly in a number of cellular processes such as DNA repair, replication and recombination, in cell division and cell differentiation or in the signalling pathways that sense alterations in the integrity of the genome. As PARP activity may significantly reduce the cellular NAD<sup>+</sup> pool, it has also been suggested that the enzyme may play a critical role in programmed cell death (Heller *et al.*, 1995; Zhang *et al.*, 1994). Further, it has been suggested that nicotinamide resulting from NAD<sup>+</sup> hydrolysis or the products of the turn-over of poly-ADP-ribose by poly-ADP-ribose glycohydrolase may be stress response signals in eukaryotes.

The information currently available on the biological function of plant PARP has come from experiments involving PARP inhibitors suggesting an *in vivo* role in the prevention of homologous recombination at sites of DNA damage as rates of homologous intrachromosomal recombination in tobacco are increased after application of 3-aminobenzamide (3ABA) (Puchta *et al.*, 1995). Furthermore, application of PARP inhibitors, such as 3ABA, nicotinamide, and 6(5H)-phenasthridinone, to differentiating cells of *Zinnia* or of *Helianthus tuberosum* has been shown to prevent development of tracheary elements (Hawkins and Phillips, 1983; Phillips and Hawkins, 1985; Shoji *et al.*, 1997; Sugiyama *et al.*, 1995), which is considered to be an example of programmed cell death in plants.

PCT application WO97/06267 describes the use of PARP inhibitors to improve the transformation (qualitatively or quantitatively) of eukaryotic cells, particularly plant cells.

Lazebnik *et al.* (1994) identified a protease with properties similar to the interleukin 1- $\beta$ -converting enzyme capable of cleaving PARP, which is an early event in apoptosis of animal cells.

Kuepper *et al.* (1990) and Molinette *et al.* (1993) have described the overproduction of the 46 kDa human PARP DNA-binding domain and various mutant forms thereof, in transfected CV-1 monkey cells or human fibroblasts and have demonstrated the trans-dominant inhibition of resident PARP activity and the consequent block of base excision DNA repair in these cells.

Ding *et al.* (1992), and Smulson *et al.* (1995) have described depletion of PARP by antisense RNA expression in mammalian cells and observed a delay in DNA strand break joining, and inhibition of differentiation of 3T3-L1 preadipocytes.

Ménissier de Murcia *et al.*, (1997) and Wang *et al.* (1995, 1997) have generated transgenic "knock-out" mice mutated in the PARP gene, indicating that PARP is not an essential protein. Cells of PARP-deficient mice are, however, more sensitive to DNA damage and differ from normal cells of animals in some aspects of induced cell death (Heller *et al.*, 1995).

### **Summary and objects of the invention.**

The invention provides a method for modulating programmed cell death in a eukaryotic cell, comprising reducing the functional level of the total PARP activity in a eukaryotic cell using the nucleotide sequence of a PARP gene of the ZAP class, and the nucleotide sequence of a PARP gene of the NAP class, preferably to reduce expression of the endogeneous PARP genes, to reduce the apparent activity of the proteins encoded by the endogenous PARP genes or to alter the nucleotide sequence of the endogenous PARP genes.

The invention also provides a method for modulating programmed cell death in a eukaryotic cell, comprising introducing a first and a second PCD modulating chimeric gene in a eukaryotic cell, preferably a plant cell, wherein the first PCD modulating

chimeric gene comprises the following operably linked DNA regions: a promoter, operative in a eukaryotic cell; a DNA region, which when transcribed yields a RNA molecule which is either capable of reducing the functional level of a Zn-finger containing PARP protein of the ZAP class; or is capable of being translated into a peptide or protein which when expressed reduces the functional level of a PARP protein of ZAP class and a DNA region involved in transcription termination and polyadenylation

and wherein the second PCD modulating chimeric gene comprises the following operably linked DNA regions :a promoter, operative in the eukaryotic cell; a DNA region, which when transcribed yields a RNA molecule which is either capable of reducing the functional level of a PARP protein of the NAP class; or capable of being translated into a peptide or protein which when expressed reduces the functional level of a PARP protein of the NAP class, and a DNA region involved in transcription termination and polyadenylation; and wherein the total apparent PARP activity in the eukaryotic cell is reduced significantly, (preferably the total apparent PARP activity is reduced from about 75% to about 90% of the normal apparent PARP activity in the eukaryotic cell, and the eukaryotic cell is protected against programmed cell death) or almost completely (preferably the total apparent PARP activity is reduced from about 90% to about 100% of the normal apparent PARP activity in the eukaryotic cell, and the cell is killed by programmed cell death).

Preferably the first transcribed DNA region or the second transcribed DNA region or both, comprise a nucleotide sequence of at least about 100 nucleotides with 75% identity to the sense DNA strand of an endogenous PARP gene of the ZAP or the NAP class, and encode a sense RNA molecule is capable of reducing the expression of the endogenous PARP gene of the ZAP or the NAP class.

In an alternative method for modulating programmed cell death, provided by the invention, the first transcribed DNA region or the second transcribed DNA region or both, comprise a nucleotide sequence of at least about 100 nucleotides with 75% identity to the complement of the sense DNA strand of an endogenous PARP gene of the ZAP or the NAP class, and encode RNA molecule is capable of reducing the expression of said endogenous PARP gene of the ZAP or the NAP class.

In yet an alternative method for modulating programmed cell death, provided by the invention, the first and/ or second transcribed DNA region encodes a RNA molecule comprising a sense nucleotide sequence of at least about 100 nucleotides with 75% identity to the mRNA resulting from transcription of an endogenous PARP gene of the ZAP or the NAP class and the RNA molecule further comprising an antisense nucleotide sequence of at least about 100 nucleotides with 75% identity to the complement of the mRNA resulting from transcription of the endogenous PARP gene of the ZAP or the NAP class, wherein the sense and antisense nucleotide sequence are capable of forming a double stranded RNA region, and wherein that RNA molecule is capable of reducing the expression of the endogenous PARP gene of the ZAP or the NAP class.

In a further alternative method for modulating programmed cell death, provided by the invention, the first and/ or second transcribed DNA region encodes a dominant negative PARP mutant capable of reducing the apparent activity of the PARP protein encoded by an endogenous PARP gene of the ZAP or the NAP class, preferably comprising amino acid sequence selected from the amino acid sequence of SEQ ID No 4 from amino acid 1 to 159 or the amino acid sequence of SEQ ID No 6 from amino acid 1 to 138 or comprising an amino acid sequence selected from the amino acid sequence of SEQ ID No 2 from amino acid 1 to 370, the amino acid sequence of SEQ ID No 11 from amino acid 1 to 98, or the amino acid sequence of SEQ ID No 2 from amino acid 1 to 370 wherein the amino acid sequence from amino acid 1 to 88 is replaced by the amino acid sequence of SEQ ID No 11.

The promoter of the first and second chimeric PCD modulating genes, or both, may be a tissue specific or inducible promoter such as a promoter is selected from a fungus-responsive promoter, a nematode-responsive promoter, an anther-selective promoter, a stigma-selective promoter, a dehiscence-zone selective promoter.

The invention also provides a method for modulating programmed cell death in a plant cell, comprising introduction of a PCD modulating chimeric gene in said plant cell, wherein the PCD modulating chimeric gene comprises the following operably linked DNA regions: a plant-expressible promoter, a DNA region, which when transcribed yields a RNA molecule, which is either capable of reducing the

expression of endogenous PARP genes; or is capable of being translated into a peptide or protein which when expressed reduces the apparent PARP activity in the plant cell, and a DNA region involved in transcription termination and polyadenylation, wherein the total apparent PARP activity in the plant cell is reduced from about 75% to about 100% of the normal apparent PARP activity in the plant cell.

It is another objective of the invention to provide the first and second chimeric PCD modulating gene as well as a eucaryotic cell, particularly a plant cell comprising the first and second chimeric PCD modulating gene and non-human eukaryotic organisms, particularly plants comprising such cells.

Finally, the invention also provides an isolated DNA sequence comprising the nucleotide sequence of SEQ ID No 1 from the nucleotide at position 113 to the nucleotide at position 3022, an isolated DNA sequence comprising the nucleotide sequence of SEQ ID No 10 from the nucleotide at position 81 to the nucleotide at position 3020 and an isolated DNA sequence comprising the nucleotide sequence of SEQ ID No 3 from the nucleotide at position 107 to the nucleotide at position 2068.

### **Brief description of the drawings**

**Figure 1.** The deduced N-terminal amino acid sequences of plant poly(ADP-ribose) polymerases.

(A) Alignment of the sequences upstream of the NAD<sup>+</sup>-binding domain found in *Arabidopsis thaliana* APP (*A.th.* APP; EMBL accession number Z48243; SEQ ID No 6) and the maize homolog NAP (*Z.m.* NAP; EMBL accession number AJ222588; SEQ ID No 4). The domain division shown is as previously proposed (Lepiniec *et al.*, 1995). The nuclear localization signal (NLS) located in the B domain is indicated by the bracket. The sequence of the B domain is not very well conserved between dicotyledonous and monocotyledonous plants. The C domain is probably comparable in function to the automodification domain of PARP from animals. The imperfect repeats, A1 and A2, are also present in maize NAP. To illustrate the internally imperfect two-fold symmetry within the repeat sequence, the properties of amino acid residues are highlighted below the sequences as follows:



filled-in circles, hydrophobic residue; open circle, glycine; (+), positively charged residue; (-), negatively charged residue; wavy line, any residue. The axis of symmetry is indicated by the vertical arrowhead and arrowhead lines mark the regions with the inverted repetition of amino acid side chain properties.

- (B) Alignment of the DNA-binding and auto-catalytic domains of mouse PARP and maize ZAP. Zn-finger-containing maize ZAP1 and ZAP2 (partial cDNA found by the 5'RACE PCR analysis) are indicated as *Z.m.* ZAP (EMBL accession number AJ222589; SEQ ID No 2) and *Z.m.* ZAP(race) (SEQ ID No 11 from amino acid at position 1 to amino acid at position 98), respectively, and the mouse PARP, *M.m.* ADPRT (Swissprot accession number P11103). The Zn-fingers and bipartite NLS of the mouse enzyme are indicated by brackets, the Caspase 3 cleavage site by the asterisk, and the putative NLS in the ZAP protein by the bracket in bold below the maize sequence. The amino acid residues that are conserved in all sequences are boxed; amino acid residues with similar physico-chemical properties are shaded with the uppermost sequence as a reference.

**Figure 2.** Comparison of the NAD<sup>+</sup>-binding domain of mouse PARP and plant PARP proteins. The range of the "PARP signature" is indicated above the sequences. Names and sequence alignment are as in Figure 1.

**Figure 3.** Estimation of the gene copy number and transcript size for the *nap* and *zap* genes.

(A) and (B) Maize genomic DNA of variety LG2080 digested with the indicated restriction endonucleases, resolved by agarose gel electrophoresis, blotted, and hybridized with radioactively labelled DNA probes prepared from the 5' domains of the *nap* and *zap* cDNA, which do not encode the NAD<sup>+</sup>-binding domain. The hybridization pattern obtained with the *nap* probe (A) is simple and indicates a single *nap* gene in the maize genome. As can be seen from the hybridization pattern (B), there might be at least two *zap* genes. To determine the size of the transcripts encoded by the *zap* and *nap* genes, approximately 1 µg of poly(A)<sup>+</sup> RNA extracted from roots (lane 1) and shoots (lane 2) of 6-day-old seedlings were resolved on an agarose gel after denaturation with glyoxal, blotted, and hybridized with *nap* (C) and *zap* (D) <sup>32</sup>P-labelled cDNA. <sup>33</sup>P 5' end-labelled *Bst*II fragments of λDNA were used as a molecular weight

markers in both DNA and RNA gel blot experiments; their positions are indicated in kb to the left of each panel.

**Figure 4.** Analysis of APP expression in yeast.

- (A) Schematic drawing of the expression cassette in pV8SPA. The expression of the app cDNA is driven by a chimeric yeast promoter, which consists of the minimal TATA box-containing promoter region of the *cycl* gene (CYC1) and an upstream activating promoter region of the *gal10* gene (GAL10), the latter providing promoter activation by galactose. Downstream regulatory sequences are derived from the gene encoding phosphoglycerol kinase (3PGK) (Kuge and Jones, 1994). The app-coding region is drawn with a division in putative domains as proposed earlier (Lepiniec *et al.*, 1995): A1 and A2 correspond to imperfect 27-amino acid repeats, in between which there is a sequence (B domain), rich in positively charged amino acids and resembling the DNA-binding domains of a number of DNA-binding proteins. The amino acid sequence of the B domain is shown below the map and the stretch of arginine and lysine residues, which may function as an NLS is drawn in bold. Methionine residues (M<sup>1</sup>, M<sup>72</sup>), which may function as translation initiation codons, are indicated above the map. The C domain is rich in glutamic acid residues, resembling in its composition, but not in its sequence, the auto-modification domain of PARP from animals.
- (B) Immunoblot (Western blot) and Northern blot analyses of the DY (pYeDP1/8-2) and DY(pV8SPA) strains, indicated as (vector) and (app), respectively. Strains were grown in SDC medium supplemented with glucose (GLU), galactose (GAL), galactose and 3mM of 3ABA (GAL+3ABA), or galactose and 5 mM nicotinamide (GAL+NIC). Total RNA or total protein were extracted from the same cultures. Ten micrograms of total protein were fractionated by electrophoresis on 10% SDS-PAGE, electroblotted, and probed with anti-APP antisera. Five micrograms of total RNA were resolved by electrophoresis on an 1.5% agarose gel, blotted onto nylon membranes, and hybridized with <sup>32</sup>P-labeled DNA fragments derived from the app cDNA. Positions of the molecular weight marker bands are indicated to the left in kilobases (kb) and kilodalton (kDa).

**Figure 5.** Poly(ADP-ribose) polymerase activity of the APP protein.

- (A) The total protein extracts were prepared from DY(pYeDP1/8-2) grown on SDC with 2% galactose (vector GAL) and DY(pV8SPA) grown either on SDC with 2% glucose (app GLU), on SDC with 2% galactose (app GAL), or on SDC with 2% galactose and 3 mM 3ABA (app GAL+3ABA). To detect the synthesis of the poly(ADP-ribose) in these extracts, samples were incubated with  $^{32}\text{P}$ -NAD<sup>+</sup> for 40 min at room temperature. Two control reactions were performed: 100 ng of the purified human PARP were incubated either in a reaction buffer alone (PARP) (lane 5), or with protein extract made from DY(pYeDP1/8-2) culture grown on glucose (vector GLU+PARP) (lane 6). The autoradiograph obtained after exposure of the dried gel to X-Omat Kodak film is shown. ORi corresponds to the beginning of the sequencing gel.
- (B) Stimulation of poly(ADP-ribose) synthesis by DNA in protein extracts from DY(pV8SPA). Amounts of sonicated salmon sperm DNA added to the nucleic acid depleted yeast extracts are indicated in  $\mu\text{g ml}^{-1}$ . The synthesis of the poly(ADP-ribose) is blocked by 3ABA, which was added in one of the reactions at a concentration of 3 mM (lane 5). To ensure the maximal recovery of the poly(ADP-ribose), 20  $\mu\text{g}$  of glycogen were included as a carrier during precipitation steps; this, as can be seen, however resulted in high carry-over of the unincorporated label.

**Figure 6.** Schematic representation of the T-DNA vectors comprising the PCD modulating chimeric genes of the invention. P35S: CaMV35S promoter; L: cab22 leader; ZAP; coding region of a PARP gene of the ZAP class; 5'ZAP: N-terminal part of the coding region of a PARP gene of the ZAP class in inverted orientation; 3' 35S: CaMV35S 3' end transcription termination signal and polyadenylation signal; pACT2: promoter region of the actin gene; pNOS; nopaline synthase gene promoter; gat: gentamycin acetyl transferase; bar: phosphinotricin acetyl transferase; 3'NOS: 3' end transcription termination signal and polyadenylation signal of nopaline synthase gene; APP: coding region of a PARP gene of the NAP class; 5'APP: N-terminal part of the coding region of a PARP gene of the NAP class in inverted orientation ; LB: left T-DNA border; RB: right T-DNA border; pTA29: tapetum specific promoter, pNTP303: pollen specific promoter.

### **Detail d d scription of preferred embodiments**

For the purpose of the invention, the term "plant-expressible promoter" means a promoter which is capable of driving transcription in a plant cell. This includes any promoter of plant origin, but also any promoter of non-plant origin which is capable of directing transcription in a plant cell, e.g., certain promoters of viral or bacterial origin such as the CaMV35S or the T-DNA gene promoters.

The term "expression of a gene" refers to the process wherein a DNA region under control of regulatory regions, particularly the promoter, is transcribed into an RNA which is biologically active i.e., which is either capable of interaction with another nucleic acid or protein or which is capable of being translated into a biologically active polypeptide or protein. A gene is said to encode an RNA when the end product of the expression of the gene is biologically active RNA, such as e.g. an antisense RNA or a ribozyme. A gene is said to encode a protein when the end product of the expression of the gene is a biologically active protein or polypeptide.

The term "gene" means any DNA fragment comprising a DNA region (the "transcribed DNA region") that is transcribed into a RNA molecule (e.g., a mRNA) in a cell under control of suitable regulatory regions, e.g., a plant-expressible promoter. A gene may thus comprise several operably linked DNA fragments such as a promoter, a 5' leader sequence, a coding region, and a 3' region comprising a polyadenylation site. An endogenous plant gene is a gene which is naturally found in a plant species. A chimeric gene is any gene which is not normally found in a plant species or, alternatively, any gene in which the promoter is not associated in nature with part or all of the transcribed DNA region or with at least one other regulatory regions of the gene.

As used herein "comprising" is to be interpreted as specifying the presence of the stated features, integers, steps or components as referred to, but does not preclude the presence or addition of one or more features, integers, steps or components, or groups thereof. Thus, e.g., a nucleic acid or protein comprising a sequence of

nucleotides or amino acids, may comprise more nucleotides or amino acids than the actually cited ones, i.e., be embedded in a larger nucleic acid or protein. A chimeric gene comprising a DNA region which is functionally or structurally defined, may comprise additional DNA regions etc.

The invention is based on the one hand on the finding that eukaryotic cells, particularly plant cells, quite particularly *Zea mays* cells contain simultaneously at least two functional major PARP protein isoforms(classes) which differ in size and amino-acid sequence, yet are both capable of binding DNA, particularly DNA with single stranded breaks, and both have poly-ADP ribosylation activity. On the other hand, the inventors have realized that programmed cell death in eukaryotes, particularly in plants, can be modulated by altering the expression level of the PARP genes or by altering the activity of the encoded proteins genetically, and that in order to achieve this goal, the expression of both genes needs to be altered or in the alternative both classes of proteins need to be altered in their activity.

Thus, the invention relates to modulation -i.e. the enhancement or the inhibition- of programmed cell death or apoptosis in eukaryotic cells, preferably plant cells, by altering the level of expression of PARP genes, or by altering the activity or apparent activity of PARP proteins in that eukaryotic cell. Conveniently, the level of expression of PARP genes or the activity of PARP proteins is controlled genetically by introduction of PCD modulating chimeric genes altering the expression of PARP genes and/or by introduction of PCD modulating chimeric genes altering the apparent activity of the PARP proteins and/or by alteration of the endogenous PARP encoding genes.

As used herein, "enhanced PCD" with regard to specified cells, refers to the death of those cells, provoked by the methods of the invention, whereby the killed cells were not destined to undergo PCD when compared to similar cells of a normal plant not modified by the methods of the invention, under similar conditions.

"Inhibited PCD" with regard to specified cells is to be understood as the process whereby a larger fraction of those cells or groups of cells, which would normally

(without the intervention by the methods of this invention) undergo programmed cell death under particular conditions, remain alive under those conditions.

The expression of the introduced PCD modulating chimeric genes or of the modified endogenous genes will thus influence the functional level of PARP protein, and indirectly interfere with programmed cell death. A moderate decrease in the functional level of PARP proteins leads to an inhibition of programmed cell death, particularly to prevention of programmed cell death, while a severe decrease in the functional level of the PARP proteins leads to induction of programmed cell death.

In accordance with the invention, it is preferred that in order to inhibit or prevent programmed cell death in a eukaryotic cell, particularly in a plant cell, the combined level of both PARP proteins and/or their activity or apparent activity is decreased significantly, however avoiding that DNA repair (governed directly or indirectly by PARP) is inhibited in such a way that the cells wherein the function of the PARP proteins is inhibited cannot recover from DNA damage or cannot maintain their genome integrity. Preferably, the level and/or activity of the PARP proteins in the target cells, should be decreased about 75 %, preferably about 80%, particularly about 90% of the normal level and/or activity in the target cells so that about 25%, preferably about 20%, particularly about 10% of the normal level and/or activity of PARP is retained in the target cells . It is further thought that the decrease in level and/or activity of the PARP proteins should not exceed 95%, preferably not exceed 90% of the normal activity and/or level in the target cells. Methods to determine the content of a specific protein such as the PARP proteins are well known to the person skilled in the art and include, but are not limited to (histochemical) quantification of such proteins using specific antibodies. Methods to quantify PARP activity are also available in the art and include the above-mentioned TUNEL assay (*in vivo*) or the *in vitro* assay described Collinge and Althaus (1994) for synthesis of poly (ADP-ribose) (see Examples).

Also in accordance with the invention, it is preferred that in order to trigger programmed cell death in a eukaryotic cell, particularly in a plant cell, the combined level of both PARP proteins and/or their activity or apparent activity is decreased substantially, preferably reduced almost completely such that the DNA repair and

maintenance of the genome integrity are no longer possible. Preferably, the combined level and/or activity of the PARP proteins in the target cells, should be decreased at least about 90%, preferably about 95%, more preferably about 99%, of the normal level and/or activity in the target cells, particularly the PARP activity should be inhibited completely. It is particularly preferred that the functional levels of both classes of PARP proteins separately are reduced to the mentioned levels.

For the purpose of the invention, PARP proteins are defined as proteins having poly (ADP-ribose) polymerase activity, preferably comprising the so-called "PARP signature". The PARP signature is an amino acid sequence which is highly conserved between PARP proteins, defined by de Murcia and Menussier de Murcia (1994) as extending from amino acid at position 858 to the amino acid at position 906 from the *Mus musculus* PARP protein. This domain corresponds to the amino acid sequence from position 817 to 865 of the conventional PARP protein of *Zea mays* (ZAP1; SEQ ID No 2) or to the amino acid sequence from position 827 to 875 of the conventional PARP protein of *Zea mays* (ZAP2; SEQ ID No 11) or to the amino acid sequence from position 500 to 547 of the non-conventional PARP protein of *Zea mays* (SEQ ID No 4) or to the amino acid sequence from position 485 to 532 of the non-conventional PARP protein of *Arabidopsis thaliana* (SEQ ID No 6). This amino sequence is highly conserved between the different PARP proteins (having about 90% to 100% sequence identity). Particularly conserved is the lysine at position 891 (corresponding to position 850 of SEQ ID No 2, position 861 of SEQ ID No 11, position 532 of SEQ ID No 4, position 517 of SEQ ID No 6) of the PARP protein from *Mus musculus*, which is considered to be involved in the catalytic activity of PARP proteins. Particularly the amino acids at position 865, 866, 893, 898 and 899 of the PARP protein of *Mus musculus* or the corresponding positions for the other sequences are variable. PARP proteins may further comprise an N-terminal DNA binding domain and/or a nuclear localization signal (NLS).

Currently, two classes of PARP proteins have been described. The first class, as defined herein, comprises the so-called classical Zn-finger containing PARP proteins (ZAP). These proteins range in size from 113-120 kDa and are further characterized by the presence of at least one, preferably two Zn-finger domains located in the N-terminal domain of the protein, particularly located within the about 355 to about 375

first amino acids of the protein. The Zn-fingers are defined as peptide sequences having the sequence CxxCx<sub>n</sub>HxxC (whereby n may vary from 26 to 30) capable of complexing a Zn atom. Examples of amino acid sequences for PARP proteins from the ZAP class include the sequences which can be found in the PIR protein database with accession number P18493 (*Bos taurus*), P26466 (*Gallus gallus*), P35875 (*Drosophila melanogaster*), P09874 (*Homo sapiens*), P11103 (*Mus musculus*), Q08824 (*Oncorhynchus masou*), P27008 (*Rattus norvegicus*), Q11208 (*Sarcophaga peregrina*), P31669 (*Xenopus laevis*) and the currently identified sequences of the ZAP1 and ZAP2 protein from *Zea mays* (SEQ ID No 2 / SEQ ID No 11).

The nucleotide sequence of the corresponding cDNAs can be found in the EMBL database under accession numbers D90073 (*Bos taurus*), X52690 (*Gallus gallus*), D13806 (*Drosophila melanogaster*), M32721 (*Homo sapiens*), X14206 (*Mus musculus*), D13809 (*Oncorhynchus masou*), X65496 (*Rattus norvegicus*), D16482 (*Sarcophaga peregrina*), D14667 (*Xenopus laevis*) and in SEQ ID No 1 and 10 (*Zea mays*).

The second class as defined herein, comprises the so-called non-classical PARP proteins (NAP). These proteins are smaller (72-73 kDa) and are further characterized by the absence of a Zn-finger domain at the N-terminus of the protein, and by the presence of an N-terminal domain comprising stretches of amino acids having similarity with DNA binding proteins. Preferably, PARP protein of these class comprise at least one amino acid sequence of about 30 to 32 amino acids which comprise the sequence R G x x x x G x K x x x x R L (amino acids are represented in the standard one-letter code, whereby x stands for any amino acid; SEQ ID No 7). Even more preferably these PARP proteins comprise at least 1 amino acid sequence of about 32 amino acids having the sequence x L x V x x x R x x L x x R G L x x x G V K x x L V x R L x x A I (SEQ ID No 8) (the so-called A1 domain) or at least 1 amino acid sequence of about 32 amino acids having the sequence G M x x x E L x x x A x x R G x x x x G x K K D x x R L x x (SEQ ID No 9) (the so-called A2 domain) or both. Particularly, the A1 and A2 domain are capable of forming a helix-loop-helix structure. These PARP proteins may further comprise a basic "B" domain (K/R rich amino acid sequence of about 35 to about 56 amino acids, involved in targeting the protein to the nucleus) and/or an acid "C" domain (D/E rich amino acid sequence of



about 36 amino acids). Examples of protein sequences from the NAP class include the APP protein from *Arabidopsis thaliana* (accessible from PIR protein database under accession number Q11207; SEQ ID No 6) and the NAP protein from *Zea mays* (SEQ ID No 4). The sequence of the corresponding cDNAs can be found in the EMBL database under accession number Z48243 (SEQ ID No 5) and in SEQ ID No 3. That the second class of PARP proteins are indeed functional PARP proteins, i.e. are capable of catalyzing DNA dependent poly(ADP-ribose) polymerization has been demonstrated by the inventors (see Example 2).

The inventors have further demonstrated that eukaryotic cells, particularly plant cells express simultaneously genes encoding PARP proteins from both classes.

It is clear that for the purpose of the invention, other genes or cDNAs encoding PARP proteins from both classes as defined, or parts thereof, can be isolated from other eukaryotic species or varieties, particularly from other plant species or varieties. These PARP genes or cDNAs can be isolated e.g. by Southern hybridization (either low-stringency or high-stringency hybridization depending on the relation between the species from which one intends to isolate the PARP gene and the species from which the probe was ultimately derived) using as probes DNA fragments with the nucleotide sequence of the above mentioned PARP genes or cDNAs, or parts thereof, preferably parts which are conserved such as a gene fragment comprising the nucleotide sequence encoding the PARP signature mentioned supra. The nucleotide sequences corresponding to the PARP signature from the PARP proteins encoded by plant genes are the nucleotide sequence of SEQ ID No 1 from nucleotide 2558 to 2704 or the nucleotide sequence of SEQ ID No 3 from nucleotide 1595 to 1747 or the nucleotide sequence of SEQ ID No 5 from nucleotide 1575 to 1724. If a discrimination is to be made between the classes of PARP genes, parts of the PARP genes which are specific for the class, such as the N-terminal domains preceding the catalytic domain or parts thereof, should preferably be used.

Alternatively, the genes or cDNAs encoding PARP proteins or parts thereof, can also be isolated by PCR-amplification using appropriate primers such as the degenerated primers with the nucleotide sequence corresponding to the sequences indicated in

SEQ ID No 13, SEQ ID No 14, or primers with the nucleotide sequence corresponding to the sequences indicated in SEQ ID No 15 to 20. However, it is clear that the person skilled in the art can design alternative oligonucleotides for use in PCR or can use oligonucleotides comprising a nucleotide sequence of at least 20, preferably at least about 30, particularly at least about 50, consecutive nucleotides of any of the PARP genes to isolate the genes or part thereof by PCR amplification.

It is clear that a combination of these techniques, or other techniques (including e.g. RACE-PCR), available to the skilled artisan to isolate genes or cDNAs on the basis of partial fragments and their nucleotide sequence, e.g. obtained by PCR amplification, can be used to isolate PARP genes, or parts thereof, suitable for use in the methods of the invention.

Moreover, PARP genes, encoding PARP proteins wherein some of the amino acids have been exchanged for other, chemically similar, amino acids (so-called conservative substitutions), or synthetic PARP genes (which encode similar proteins as natural PARP genes but with a different nucleotide sequence, based on the degeneracy of the genetic code) and parts thereof are also suited for the methods of the invention.

In one aspect of the invention, PCD in eukaryotic cells, particularly in plant cells, is inhibited by a moderate decrease in the functional level of PARP in those eukaryotic cells.

In one embodiment of this first aspect of the invention, the functional level of PARP in eukaryotic cells, particularly in plant cells is reduced by introduction of at least one PCD modulating chimeric gene in those cells, comprising a promoter capable of directing transcription in these cells, preferably a plant-expressible promoter, and a functional 3' transcription termination and polyadenylation region, operably linked to a DNA region which when transcribed yields a biologically active RNA molecule which is capable of decreasing the functional level of the endogenous PARP activity encoded by both classes of PARP genes.

In a preferred embodiment, at least two such PCD modulating chimeric genes are introduced in the cells, whereby the biologically active RNA encoded by the first PCD modulating chimeric gene decreases the functional level of the endogenous PARP activity encoded by the genes of the NAP class, and whereby the biologically active RNA encoded by the second PCD modulating chimeric gene decreases the functional level of the endogenous PARP activity encoded by the genes of the ZAP class, so that the combined PARP activity is moderately decreased.

In a particularly preferred embodiment, the PCD modulating chimeric genes decrease the functional level of the endogenous PARP activity by reducing the level of expression of the endogenous PARP genes. To this end, the transcribed DNA region encodes a biologically active RNA which decreases the mRNAs encoding NAP and ZAP class PARP proteins, that is available for translation. This can be achieved through techniques such as antisense RNA, co-suppression or ribozyme action.

As used herein, "co-suppression" refers to the process of transcriptional and/or post-transcriptional suppression of RNA accumulation in a sequence specific manner, resulting in the suppression of expression of homologous endogenous genes or transgenes.

Suppressing the expression of the endogenous PARP genes can thus be achieved by introduction of a transgene comprising a strong promoter operably linked to a DNA region whereby the resulting transcribed RNA is a sense RNA or an antisense RNA comprising a nucleotide sequence which has at least 75%, preferably at least 80%, particularly at least 85%, more particularly at least 90%, especially at least 95% sequence identity with or is identical to the coding or transcribed DNA sequence (sense) or to the complement (antisense) of part of the PARP gene whose expression is to be suppressed. Preferably, the transcribed DNA region does not code for a functional protein. Particularly, the transcribed region does not code for a protein. Further, the nucleotide sequence of the sense or antisense region should preferably be at least about 100 nucleotides in length, more preferably at least about 250 nucleotides, particularly at least about 500 nucleotides but may extend to the full length of the coding region of the gene whose expression is to be reduced.

For the purpose of this invention the "sequence identity" of two related nucleotide or amino acid sequences, expressed as a percentage, refers to the number of positions in the two optimally aligned sequences which have identical residues (x100) divided by the number of positions compared. A gap, i.e. a position in an alignment where a residue is present in one sequence but not in the other is regarded as a position with non-identical residues. The alignment of the two sequences is performed by the Wilbur and Lipmann algorithm (Wilbur and Lipmann, 1983) using a window-size of 20 nucleotides or amino acids, a word length of 2 amino acids, and a gap penalty of 4. Computer-assisted analysis and interpretation of sequence data, including sequence alignment as described above, can be conveniently performed using commercially available software packages such as the programs of the Intelligenetics<sup>TM</sup> Suite (Intelligenetics Inc., CA).

It will be clear to a skilled artisan that one or more sense or antisense PCD modulating chimeric genes can be used to achieve the goals of the first aspect of the invention. When one sense or antisense PCD modulating chimeric gene is used, this gene must be capable of simultaneously reducing the expression of PARP genes of both classes. This can e.g. be achieved by choosing the transcribed region of the chimeric gene in such a way that expression of both classes of genes can be regulated by one sense or antisense RNA, i.e. by choosing target regions corresponding to the highest homology DNA region of the PARP genes of both classes and incorporating a sense or antisense transcribed DNA region corresponding to both target regions, conform to the conditions described above for sense and antisense RNA. Alternatively, different sense or antisense RNA regions, each specific for regulating the expression of one class of PARP genes, can be combined into one RNA molecule, encoded by one transcribed region of one PCD modulating chimeric gene. Obviously, the different sense or antisense RNA regions specific for regulating the expression of one class of PARP genes can be introduced as separate PCD modulating chimeric genes.

Preferred sense and antisense encoding transcribed regions comprise a nucleotide sequence corresponding (with sequence identity constraints as indicated above) to a sequence of at least about 100 consecutive nucleotides selected from the N-terminal

domains of the PARP genes, preferably corresponding to a sequence of at least about 100 consecutive nucleotides selected from the sequence of SEQ ID No 1 from nucleotide position 113 to 1189, the sequence of SEQ ID No 3 from nucleotide position 107 to 583, the sequence of SEQ ID No 5 from nucleotide position 131 to 542 or the sequence of SEQ ID No 10 from nucleotide position 81 to 1180. However, it is clear that sense or antisense encoding transcribed regions can be used comprising a sequence corresponding to the complete sequence of the N-terminal domain of the PARP genes, or even to complete sequence of the PARP genes, particularly the protein-encoding region thereof. Further preferred are sense and antisense encoding transcribed regions which comprise a nucleotide sequence corresponding (with sequence identity constraints as indicated above) to a sequence of at least about 100 consecutive nucleotides selected from the C-terminal catalytic domains of the PARP genes, preferably a sequence of at least 100 nucleotides encompassing the PARP-signature encoding nucleotide sequences, particularly the PARP-signature encoding nucleotide sequences indicated *supra*. Again, it is clear that sense or antisense encoding transcribed regions can be used comprising a sequence corresponding to the complete sequence of the C-terminal domain of the PARP genes.

In another particularly preferred embodiment, the PCD modulating chimeric genes decrease the functional level of the endogenous PARP activity by reducing the level of apparent activity of the endogenous PARPs of both classes. To this end, the transcribed DNA region encodes a biologically active RNA which is translated into a protein or inhibiting NAP or ZAP class PARP proteins or both, such as inactivating antibodies or dominant negative PARP mutants.

"Inactivating antibodies of PARP proteins" are antibodies or parts thereof which specifically bind at least to some epitopes of PARP proteins, such as the epitope covering part of the ZN finger II from position 111-118 in ZAP1 or a corresponding peptide in ZAP2, and which inhibit the activity of the target protein.

"Dominant negative PARP mutants" as used herein, are proteins or peptides comprising at least part of a PARP protein (or a variant thereof), preferably a PARP protein endogenous to the eukaryotic target host cell, which have no PARP activity,

and which have an inhibitory effect on the activity of the endogenous PARP proteins when expressed in that host cell. Preferred dominant negative PARP mutants are proteins comprising or consisting of a functional DNA binding domain (or a variant thereof) without a catalytic domain (such as the N-terminal Zn-finger containing domain of about 355 to about 375 amino acids of a PARP of the ZAP class, particularly a DNA binding protein domain comprising the amino acid sequence of SEQ ID No 2 from amino acid 1 to 370 or a DNA binding protein domain comprising the amino acid sequence of SEQ ID No 11 from amino acid 1 to 98, or a DNA binding protein domain comprising the amino acid sequence of SEQ ID No 2 from amino acid 1 to 370 wherein the amino acid sequence from amino acid 1 to 88 is replaced by the amino acid sequence of SEQ ID No 11 from amino acid at position 1 to the amino acid at position 98, or such as the N-terminal DNA binding protein domain of about 135 to 160 amino acids of a PARP of the NAP class, particularly a DNA binding protein domain comprising the amino acid sequence of SEQ ID No 4 from amino acid 1 to 159 or a DNA binding protein domain comprising the amino acid sequence of SEQ ID No 6 from amino acid 1 to 138) or without a functional catalytic domain (such as inactive PARP mutants, mutated in the so-called PARP signature, particularly mutated at the conserved lysine of position 850 of SEQ ID No 2, position 532 of SEQ ID No 4, position 517 of SEQ ID No 6). Preferably, dominant negative PARP mutants should retain their DNA binding activity. Dominant negative PARP mutants can be fused to a carrier protein, such as a  $\beta$ -glucuronidase (SEQ ID No 12).

Again, one or more PCD modulating genes encoding one or more dominant negative PARP mutants can be used to achieve the goals of the first aspect of the invention. When one PCD modulating chimeric gene is used, this gene must be capable of simultaneously reducing the expression of PARP genes of both classes.

In another embodiment of the first aspect of the invention, the functional level of PARP in eukaryotic cells, particularly in plant cells is reduced by modification of the nucleotide sequence of the endogenous PARP genes in those cells so that the encoded mutant PARP proteins retain about 10% of their activity. Methods to achieve such a modification of endogenous PARP genes include homologous

recombination to exchange the endogenous PARP genes for mutant PARP genes e.g. by the methods described in US patent 5,527,695. In a preferred embodiment such site-directed modification of the nucleotide sequence of the endogenous PARP genes is achieved by introduction of chimeric DNA/RNA oligonucleotides as described in WO 96/22364 or US patent 5,565,350.

In another aspect of the invention, programmed death of eukaryotic cells, preferably selected cells, particularly selected plant cells is enhanced by a severe decrease in the functional level of PARP, preferably reduced almost completely, such that the DNA repair and maintenance of the genome integrity are no longer possible.

In one embodiment of this aspect of the invention, the functional level of PARP in eukaryotic cells, particularly in plant cells is reduced severely, particularly abolished almost completely, by introduction of at least one PCD modulating chimeric gene in those cells, comprising a promoter capable of directing transcription in these cells, preferably a plant-expressible promoter, and a functional 3' transcription termination and polyadenylation region, operably linked to a DNA region which when transcribed yields a biologically active RNA molecule which is capable of decreasing the functional level of the endogenous PARP activity encoded by both classes of PARP genes.

In a preferred embodiment of the second aspect of the invention, at least two such PCD modulating chimeric genes are introduced in the cells, whereby the biologically active RNA encoded by the first PCD modulating chimeric gene decreases the functional level of the endogenous PARP activity encoded by the genes of the NAP class, and whereby the biologically active RNA encoded by the second PCD modulating chimeric gene decreases the functional level of the endogenous PARP activity encoded by the genes of the ZAP class, so that the combined PARP activity is severely decreased, particularly almost completely eliminated.

As mentioned for the first aspect of this invention, the transcribed regions of the PCD modulating chimeric genes encode biologically active RNA, which can interfere with the expression of the endogenous PARP genes (e.g. through antisense action, co-suppression or ribozyme action) or the biologically active RNA can be further

translated into a peptide or protein, capable of inhibiting the PARP proteins of the NAP and ZAP class, such as inactivating antibodies or dominant negative PARP mutants.

In a particularly preferred embodiment of the second aspect of the invention, the transcribed region of the PCD modulating chimeric genes (PCD enhancing chimeric genes) codes for a biologically active RNA which comprises at least one RNA region (preferably of at least about 100 nucleotides in length) classifying according to the above mentioned criteria as a sense RNA for at least one of the endogenous PARP genes, and at least one other RNA region (preferably of at least about 100 nucleotides in length), classifying according to the above mentioned criteria as an antisense RNA for at least one of the endogenous PARP genes, whereby the antisense and sense RNA region are capable of combining into a double stranded RNA region (preferably over a distance of at least about 100 nucleotides). In an especially preferred embodiment, two such PCD modulating genes, one targeted to reduce the functional level of a PARP protein of the NAP class, and the other targeted to reduce the functional level of a PARP protein of the ZAP class are introduced into an eukaryotic cell or organism, preferably a plant cell or plant.

It is clear that the different embodiments for the transcribed DNA regions of the chimeric PCD modulating genes of the invention can be used in various combinations to arrive at the goals of the invention. E.g. a first chimeric PCD modulating gene may encode a sense RNA designed to reduce the expression of an endogenous PARP gene of the ZAP class, while the second chimeric PCD modulating gene may encode a dominant negative PARP mutant designed to reduce the expression of an endogenous PARP gene of the NAP class.

Whether the introduction of PCD modulating chimeric genes into eukaryotic cells will ultimately result in a moderately reduced or a severely reduced functional level of combined PARP in those cells -i.e. in inhibited PCD or enhanced PCD- will usually be determined by the expression level (either on transcriptional level or combined transcriptional/translational level) of those PCD modulating genes. A major contributing factor to the expression level of the PCD modulating gene is the choice of the promoter region, although other factors (such as, but not limited to, the choice



of the 3' end, the presence of introns, codon usage of the transcribed region, mRNA stability, presence of consensus sequence around translation initiation site, choice of 5' and 3' untranslated RNA regions, presence of PEST sequences, the influence of chromatin structure surrounding the insertion site of a stably integrated PCD modulating gene, copy number of the introduced PCD modulating genes, etc.) or combinations thereof will also contribute to the ultimate expression level of the PCD modulating gene. In general, it can be assumed that moderate reduction of functional levels of combined PARP can be achieved by PCD modulating genes comprising a relatively weak promoter, while severe reduction of functional levels of combined PARP can be achieved by PCD modulating genes comprising a relatively strong promoter. However, the expression level of a PCD modulating gene comprising a specific promoter and eventually its effect on PCD, can vary as a function of the other contributing factors, as already mentioned.

For the purpose of particular embodiments of the invention, the PCD modulating chimeric genes may comprise a constitutive promoter, or a promoter which is expressed in all or the majority of the cell types throughout the organism, particularly throughout the plant, such as the promoter regions derived from the T-DNA genes, particularly the opine synthase genes of *Agrobacterium* Ti- or Ri-plasmids (e.g. nos, ocs promoters), or the promoter regions of viral genes (such as CaMV35S promoters, or variants thereof).

It may be further advantageous to control the expression of the PCD modulating gene at will or in response to environmental cues, e.g. by inclusion of an inducible promoter which can be activated by an external stimuli, such as, but not limited to application of chemical compounds (e.g. safeners, herbicides, glucocorticoids), light conditions, exposure to abiotic stress (e.g. wounding, heavy metals, extreme temperatures, salinity or drought) or biotic stress (e.g. pathogen or pest infection including infection by fungi, viruses, bacteria, insects, nematodes, mycoplasmas and mycoplasma like organisms etc.). Examples of plant-expressible inducible promoters suitable for the invention are: nematode inducible promoters (such as disclosed in WO 92/21757), fungus inducible promoters (WO 93/19188, WO 96/28561), promoters inducible after application of glucocorticoids such as dexamethasone ( ),

or promoters repressed or activated after application of tetracyclin (Gatz *et al.* 1988 ; Weimann *et al.* 1994)

In several embodiments of the invention, particularly for the second aspect of the invention (i.e. enhanced PCD), it may be convenient or required to restrict the effect on programmed cell death to a particular subset of the cells of the organism, particularly of the plant, hence the PCD modulating genes may include tissue-specific or cell type-specific promoters. Examples of suitable plant-expressible promoters selectively expressed in particular tissues or cell types are well known in the art and include but are not limited to seed-specific promoters (e.g. WO89/03887), organ-primordia specific promoters (An *et al.*, 1996), stem-specific promoters (Keller *et al.*, 1988), leaf specific promoters (Hudspeth *et al.*, 1989), mesophyll-specific promoters (such as the light-inducible Rubisco promoters), root-specific promoters (Keller *et al.*, 1989), tuber-specific promoters (Keil *et al.*, 1989), vascular tissue specific promoters (Peleman *et al.*, 1989), meristem specific promoters (such as the promoter of the *SHOOTMERISTEMLESS* (*STM*) gene, Long *et al.*, 1996), primordia specific promoter (such as the promoter of the *Antirrhinum* CycD3a gene, Doonan *et al.*, 1998), anther specific promoters (WO 89/10396, WO9213956, WO9213957) stigma-specific promoters (WO 91/02068), dehiscence-zone specific promoters (WO 97/13865), seed-specific promoters (WO 89/03887) etc.

Preferably the chimeric PCD modulating genes of the invention are accompanied by a marker gene, preferably a chimeric marker gene comprising a marker DNA that is operably linked at its 5' end to a plant-expressible promoter, preferably a constitutive promoter, such as the CaMV 35S promoter, or a light inducible promoter such as the promoter of the gene encoding the small subunit of Rubisco; and operably linked at its 3' end to suitable plant transcription 3' end formation and polyadenylation signals. It is expected that the choice of the marker DNA is not critical, and any suitable marker DNA can be used. For example, a marker DNA can encode a protein that provides a distinguishable "color" to the transformed plant cell, such as the A1 gene (Meyer *et al.*, 1987) or Green Fluorescent Protein (Sheen *et al.*, 1995), can provide herbicide resistance to the transformed plant cell, such as the *bar* gene, encoding resistance to phosphinothricin (EP 0,242,246), or can provided antibiotic resistance

to the transformed cells, such as the *aac(6')* gene, encoding resistance to gentamycin (WO94/01560).

Methods to introduce PCD modulating chimeric genes into eukaryotic cells, particularly methods to transform plant cells are well known in the art, and are believed not to be critical for the methods of the invention. Transformation results in either transient or stably transformed cells (whereby the PCD modulating chimeric genes are stably inserted in the genome of the cell, particularly in the nuclear genome of the cell).

It is clear that the methods and means described in this invention to alter the programmed cell death in eukaryotic cells and organisms, particularly in plant cells and plants, has several important application possibilities. Inhibition of PCD by the methods and means of the invention, can be used to relieve the stress imposed upon the cells, particularly the plant cells, during transformation and thus to increase transformation efficiency, as described in WO 97/06267. Inhibition of PCD can also be used to improve cell culture of eukaryotic cells, particularly of plant cells. Triggering of PCD in particular cell types using the means and methods of the invention, can be used for methods which call upon the use of a cytotoxin. Since PCD is the "natural" way for cells to die, the use of PCD enhancing chimeric genes of the invention constitutes an improvement over the use of other cytotoxic genes such as RNase or diphtheria toxin genes which lead to cell lysis. Moreover, low-level expression of PCD enhancing genes in cells different than the targeted cells, will lead to a moderate reduction instead of a severe reduction of PARP activity in those cells, thus actually inhibiting PCD in non-target cells.

For plants, preferred applications of PCD enhancing chimeric genes include, but are not limited to:

1. the generation of plants protected against fungus infection, whereby the PCD enhancing chimeric gene or genes comprise a fungus-responsive promoter as described in WO 93/19188 or WO 96/28561.
2. the generation of nematode resistant plants, whereby the PCD enhancing chimeric gene or genes comprise a nematode inducible promoters such as disclosed in WO 92/21757

3. the generation of male or female sterile plants, whereby the PCD enhancing chimeric gene or genes comprise anther-specific promoters (such as disclosed in WO 89/10396, WO9213956, WO9213957) or stigma-specific promoters (such as disclosed in WO 91/02068)
4. the generation of plants with improved seed shatter characteristics whereby the PCD enhancing chimeric gene or genes comprise dehiscence zone-specific promoters (such as disclosed in WO 97/13865).

Although it is clear that the invention can be applied essentially to all plant species and varieties, the invention will be especially suited to alter programmed cell death in plants with a commercial value. Particularly preferred plants to which the invention can be applied are corn, oil seed rape, linseed, wheat, grasses, alfalfa, legumes, a brassica vegetable, tomato, lettuce, cotton, rice, barley, potato, tobacco, sugar beet, sunflower, and ornamental plants such as carnation, chrysanthemum, roses, tulips and the like.

The obtained transformed plant can be used in a conventional breeding scheme to produce more transformed plants with the same characteristics or to introduce the chimeric cell-division controlling gene of the invention in other varieties of the same or related plant species. Seeds obtained from the transformed plants contain the PCD modulating gene of the invention as a stable genomic insert.

The following non-limiting Examples describe the construction of chimeric apoptosis controlling genes and the use of such genes for the modulation of the programmed cell death in eukaryotic cells and organisms. Unless stated otherwise in the Examples, all recombinant DNA techniques are carried out according to standard protocols as described in Sambrook *et al.* (1989) *Molecular Cloning: A Laboratory Manual*, Second Edition, Cold Spring Harbor Laboratory Press, NY and in Volumes 1 and 2 of Ausubel *et al.* (1994) *Current Protocols in Molecular Biology*, *Current Protocols*, USA. Standard materials and methods for plant molecular work are described in *Plant Molecular Biology Labfax* (1993) by R.D.D. Croy, jointly published by BIOS Scientific Publications Ltd (UK) and Blackwell Scientific Publications, UK.

Throughout the description and Examples, reference is made to the following sequences:

SEQ ID No 1: DNA sequence of the ZAP gene of *Zea mays* (*zap1*)

SEQ ID No 2: protein sequence of the ZAP protein of *Zea mays* (ZAP1)

SEQ ID No 3: DNA sequence of the NAP gene of *Zea mays* (*nap*)

SEQ ID No 4: protein sequence of the NAP protein of *Zea mays* (NAP)

SEQ ID No 5: DNA sequence of the NAP gene of *Arabidopsis thaliana* (*app*)

SEQ ID No 6: protein sequence of the NAP protein of *Arabidopsis thaliana* (APP)

SEQ ID No 7: consensus sequence for the A domain of non-conventional PARP proteins

SEQ ID No 8: consensus sequence for the A1 domain of non-conventional PARP proteins

SEQ ID No 9: consensus sequence for the A2 domain of non-conventional PARP proteins

SEQ ID No 10: DNA sequence of the second ZAP gene of *Zea mays* (*Zap2*)

SEQ ID No 11: protein sequence of the ZAP protein of *Zea mays* (ZAP2)

SEQ ID No 12: amino acid sequence of a fusion protein between the DNA binding domain of APP and the GUS protein

SEQ ID No 13: degenerated PCR primer

SEQ ID No 14: degenerated PCR primer

SEQ ID No 15: PCR primer

SEQ ID No 16: PCR primer

SEQ ID No 17: PCR primer

SEQ ID No 18: PCR primer

SEQ ID No 19: PCR primer

SEQ ID No 20: PCR primer

SEQ ID No 21: app promoter-gus translational fusion

Sequence listing free text

The following free text has been used in the Sequence Listing part of this application

<223> Description of Artificial Sequence: A domain of

"

non-conventional PARP proteins

<223> Description of Artificial Sequence:A1 domain on  
non conventional PARP protein

<223> Description of Artificial Sequence: A2 domain of

"

non-conventional PARP protein

"

<223> Description of Artificial Sequence: fusion protein  
between APP N-terminal domain and GUS protein

<223> Description of Artificial Sequence: degenerated

"

PCR primer

<223> Description of Artificial Sequence:oligonucleotide

"

for use as PCR primer

"

<223> Description of Artificial Sequence: APP promoter  
fusion with beta-glucuronidase gene

<223> translation initiation codon

## Examples

### Experimental procedures

#### *Yeast and bacterial strains*

*Saccharomyces cerevisiae* strain DY (MATa *his3 can1-10 ade2 leu2 trp1 ura3::(3xSV40 AP1-lacZ)* (Kuge and Jones, 1994) was used for the expression of the APP protein. Yeast transformation was carried out according to Dohmen *et al.* (1991). Strains were grown on a minimal SDC medium (0.67% yeast nitrogen base, 0.37% casamino acids, 2% glucose, 50 mg l<sup>-1</sup> of adenine and 40 mg l<sup>-1</sup> of tryptophan). For the induction of the APP expression, glucose in SDC was substituted with 2% galactose.

*Escherichia coli* strain XL-1 (Stratagene, La Jolla, CA) was used for the plasmid manipulations and library screenings, which were carried out according to standard procedures (Ausubel *et al.*, 1987; Sambrook *et al.*, 1989). *E. coli* BL21 (Studier and Moffat, 1986) was used for the APP protein expression and *Agrobacterium tumefaciens* C58C1Rif<sup>R</sup>(pGV2260) (Deblaere *et al.*, 1985) for the stable transformation of plants.

#### *Poly(ADP-ribose)polymerase activity assay*

Enzymatic activity of the APP was assayed in total protein extracts of yeast strains prepared as follows. DY(pV8SPA) or DY(pYeDP1/8-2) were grown in 50 ml of SDC medium overnight at 30°C on a gyratory shaker at 150 rpm. Yeast cells were harvested by centrifugation at 1,000×g, washed three times with 150 ml of 0.1 M potassium phosphate buffer (pH 6.5), and resuspended in 5 ml of sorbitol buffer (1.2 M sorbitol, 0.12 M K<sub>2</sub>HPO<sub>4</sub>, 0.033 M citric acid, pH 5.9). Lyticase (Boehringer, Mannheim, Germany) was added to the cell suspension to a final concentration of 30 U ml<sup>-1</sup> and cells were incubated at 30°C for 1 h. Yeast spheroplasts were then washed three times with sorbitol buffer and resuspended in 2 ml of ice-cold lysis buffer (100 mM Tris-HCl, pH 7.5, 400 mM NaCl, 1 mM EDTA, 10% glycerol, 1 mM DTT). After sonication, the lysate was centrifuged at 20,000×g for 20 min at 4°C and the

supernatant was desalted on a Econo-Pack™ 10 DG column (Bio-Rad, Richmond, CA) equilibrated with reaction buffer (100 mM Tris-HCl, pH 8.0, 10 mM MgCl<sub>2</sub>, 1 mM DTT). To reduce proteolytic degradation of proteins, the lysis and reaction buffers were supplemented with a protease inhibitor cocktail (Boehringer), one tablet per 50 ml. Nucleic acids were removed from the total extracts by adding NaCl and protamine sulfate to a final concentration of 600 mM and 10 mg ml<sup>-1</sup>, respectively. After incubation at room temperature for 10 min, the precipitate was removed by centrifugation at 20,000×g for 15 min at 4°C. The buffer of the supernatant was exchanged for the reaction buffer by gel filtration on an Econo-Pack™ 10 DG column.

The assay for the synthesis of poly(ADP-ribose) was adapted from Collinge and Althaus (1994). Approximately 500 µg of total yeast protein were incubated in a reaction buffer supplemented with 30 µCi of <sup>32</sup>P-NAD<sup>+</sup> (500 Ci mmol<sup>-1</sup>), unlabeled NAD<sup>+</sup> to a final concentration of 60 µM, and 10 µg ml<sup>-1</sup> sonicated salmon sperm DNA. After incubation for 40 min at room temperature, 500 µl of the stop buffer (200 mM Tris-HCl, pH 7.6, 0.1 M NaCl, 5 mM EDTA, 1% Na<sup>+</sup>-N-lauroyl-sarcosine, and 20 µg ml<sup>-1</sup> proteinase K) were added and reactions incubated at 37°C overnight. After phenol and phenol/chloroform extractions, polymers were precipitated with 2.5 volumes of ethanol with 0.1 M NaAc (pH 5.2). The pellet was washed with 70% ethanol, dried, and dissolved in 70% formamide, 10 mM EDTA, 0.01% bromophenol blue, and 0.01% xylene cyanol. Samples were heated at 80°C for 10 min and then loaded onto a 12% polyacrylamide/6 M urea sequencing gel. Gels were dried on 3MM paper (Whatman International, Maidstone, UK) and exposed either to Kodak X-Omat X-ray film (Eastman Kodak, Richmond, NY) or scanned using a PhosphorImager™ 445SI (Molecular Dynamics, Sunnyvale, CA).

### *Immunological techniques*

A truncated *app* cDNA encoding an APP polypeptide from amino acids Met<sup>310</sup> to His<sup>637</sup> was expressed as a translation fusion with six histidine residues at the N terminus after induction of a 500-ml culture of the *E. coli* BL21(pETΔNdeSPA) with 1 mM isopropyl-β-D-thiogalactopyranoside. The APP polypeptide was purified to near homogeneity by affinity chromatography under denaturing conditions (in the presence



of 6 M guanidinium hydrochloride) on a  $\text{Ni}^{2+}$ -NTA-agarose column, according to the manufacturer's protocol (Qiagen, Chatsworth, CA). After dialysis against PBS, a mixture of the soluble and insoluble APP polypeptides was used to immunize two New Zealand White rabbits following a standard immunization protocol (Harlow and Lane, 1988). For the Western blot analysis, proteins were resolved by denaturing SDS-PAGE (Sambrook *et al.*, 1989; Harlow and Lane, 1988) and transferred onto nitrocellulose membranes (Hybond-C; Amersham), using a Semi-Dry Blotter II (Kem-En-Tec, Copenhagen, Denmark).

*In situ* antigen localization in yeast cells was carried out as described (Harlow and Lane, 1988). For the localization of the APP protein in yeast spheroplasts, anti-APP serum was diluted 1:3,000 to 1:5,000 in Tris-buffered saline-BSA buffer. 10H monoclonal antibody, which specifically recognizes poly(ADP-ribose) polymer (Ikajima *et al.*, 1990) was used in a 1:100 dilution in PBS buffer. The mouse antibody were detected with the sheep anti-mouse IgG  $\text{F(ab')}_2$  fragment conjugated to fluorescein isothiocyanate (FITC) (Sigma) at a dilution of 1:200. Rabbit IgG was detected with CY-3 conjugated sheep anti-rabbit IgG sheep  $\text{F(ab')}_2$  fragment (Sigma), at a dilution of 1:200. For the visualization of DNA, slides were incubated for 1 min in PBS with  $10 \mu\text{g ml}^{-1}$  of 4',6-diamidino-2-phenylindole (DAPI; Sigma). Fluorescence imaging was performed on an Axioskop epifluorescence microscope (Zeiss, Jena, Germany). For observation of FITC and CY-3 fluorochromes, 23 and 15 filter cubes were used, respectively. Cells were photographed with Fuji Color-100 super plus film.

#### *Plant material and histochemical analysis*

*Nicotiana tabacum* SR1 (Maliga *et al.*, 1975) was used for the generation of stable transformants following the procedure of leaf disc cocultivation (De Block *et al.*, 1987) with *A. tumefaciens* C58C1Rif<sup>R</sup>(pGV2260; pGCNSPAGUS). *N. tabacum* SR1 line transformed with authentic GUS under the control of the 35S CaMV was used as a control. *Arabidopsis thaliana* ecotype Columbia was used for the transformation of the *app*-promoter-GUS fusion following the *in situ* infiltration procedure.

For *in situ* histochemical staining of the GUS activity, plant samples were fixed in ice-cold 90% acetone for 30 min, washed in 0.1 M  $\text{K}_2\text{HPO}_4$  (pH 7.8), and then

incubated in staining buffer (0.1 M  $\text{K}_2\text{HPO}_4$ , pH 7.8, 2 mM X-Gluc, 20 mM  $\text{Fe}^{3+}$ -EDTA) at 37°C. Stained plant tissues were stored in 70% ethanol at 4°C. When necessary, browning of tissues due to phenolic oxidation was reduced by incubation with lactophenol (Beeckman and Engler, 1994). The GUS staining was examined under a Jenalumar light microscope (Zeiss). Plant tissues were photographed with Fuji Color-100 super plus film.

### *Miscellaneous methods*

The plasmid construction steps were routinely verified by DNA sequencing carried out according to protocols provided by USB Biochemicals (Cleveland, OH).  $^{32}\text{P}$ -labeled DNA probes for nucleic acid hybridization were synthesized by the Ready-Prime DNA labelling kit (Amersham). For DNA and RNA hybridization experiments, the buffer system of Church and Gilbert (1984) was used (0.25 M sodium phosphate, pH 7.2, 7% SDS, 1% BSA, 1 mM EDTA). For Western blot analysis, yeast total proteins were extracted with phenol essentially as described for plant tissues (Hurkman and Tanaka, 1986). For Northern blot analysis, total yeast RNA was extracted with hot phenol as described (Ausubel *et al.*, 1987). RNA was resolved on 1.5% agarose gels after denaturation with glyoxal (Sambrook *et al.*, 1989). Hybond-N nylon filters (Amersham) were used for the nucleic acid blotting.

### **Example 1: Isolation of genes encoding PARP homologues from *Zea mays*.**

With the purpose of isolating maize cDNA encoding PARP homologue(s) two approaches were followed. First, a maize cDNA library was screened under low-stringency DNA—DNA hybridization conditions using a DNA probe prepared from the *Arabidopsis app* cDNA. Secondly, PCR amplification of part of the maize PARP was performed, using the first-strand cDNA as a template and two degenerate primers, designed on the basis of the sequence of the "PARP signature", the most conserved amino acid sequence between all known PARP proteins.

A  $\lambda$ ZAP (Stratagene) cDNA library from leaves of maize (*Zea mays* L.), inbred line B734 . Plaques (500,000) were screened according to standard procedures (Sambrook *et al.*, 1989). After screening with the *Arabidopsis app* probe, one

non-full-length cDNA of 1.4 kbp was purified. After the initial cDNA library screening with the *app* probe and a subsequent 5' rapid amplification of cDNA ends (RACE) PCR analysis, the *nap* gene, a maize homologue of the *Arabidopsis app*, was identified. For the 5'RACE PCR, the template was prepared with the Marathon kit (Clontech, Palo Alto, CA) and 0.5 µg of maize poly(A)<sup>+</sup> RNA isolated from inner sheath, outer sheath, and leaves of 1-week-old maize seedlings. The gene-specific, nested primers for PCR amplification were 5'-GGGACCATGTAGTTTATCTTGACCT-3' (SEQ ID No 15) and 5'-GACCTCGTACCCCAACTCTTCCCAT-3' (SEQ ID No 16) for *nap* primers. The amplified PCR products were subcloned and sequenced. A fragment of 800 bp was amplified with *nap*-specific primers which allowed to reconstruct the 2295-bp-long sequence of *nap* cDNA (SEQ ID No 3).

The NAP protein was 653 amino acids long (molecular mass ~73 kDa; SEQ ID No 4) and highly similar (61% sequence identity and 69% similarity) to the APP. Most importantly, NAP had an organization of the N-terminus congruent to APP (Figure 1A), suggesting a rather strict selection pressure on the structure of APP-like proteins in plants. The *nap* gene was unique in the maize genome (Figure 2A) and encoded a transcript of 2.4 kb (Figure 2C).

Using degenerate primers based on very highly conserved regions in the "PARP signature" and first-strand cDNA from *Zea mays* as a template, a 310-bp fragment was amplified. For the PCR with degenerate primers 5'-CCGAATTCGGNTAYATGTTYGGNAA-3' (SEQ ID No 13) and 5'-CCGAATTCACNATRTAYTCRTTTRTA-3' (SEQ ID No 14) with Y=C/T; R=A/G; N=A/G/C/T), the first strand cDNA was used as a template and was synthesized using 5 µg of poly(A)<sup>+</sup> RNA from young maize leaves and MuMLV reverse transcriptase. PCR amplifications were performed with *Taq* DNA polymerase in 100 µl volume using the following conditions: 1 min at 95°C, 2 min at 45°C, 3 min at 72°C, followed by 38 cycles of 1 min at 95°C, 2 min at 45°C, 3 min at 72°C, with a final incubation for 10 min at 72°C.

The sequence of the 310 bp fragment showed 55% sequence identity and 64% sequence similarity with human PARP over the same region, but was, however, different from the sequence of the *nap* cDNA. Three *zap* cDNAs were identified after screening with the 310-bp fragment, which was obtained by PCR with degenerate primers. These three purified cDNA were all derived from the same transcript because they had identical 3' non-coding regions; the longest clone (#9) was sequenced on both strands (SEQ ID No 1). This cDNA encoded a PARP-homologous polypeptide of 689 amino acids (SEQ ID No 2; molecular mass ~109 kDa), which we designated as ZAP1 (Figure 1B). The first Zn-finger of ZAP1 was probably nonfunctional because it had the sequence CKSCxxxHASV, which included no third cysteine residue.

5'RACE PCR analysis of *zap* transcripts from the maize line LG2080 (the screened cDNA library was made from the inbred line B734) was performed as described above using the following *zap* specific primers 5'-AAGTCGACGCGGCCGCCACACCTAGTGCCAGGTCAG-3' (SEQ ID No 17) and 5'-ATCTCAATTGTACATTTCTCAGGA-3' (SEQ ID No 18). A 450-bp PCR product was obtained after PCR with *zap*-specific primers. Eight independent, because of their slight differences in lengths at their 5' ends, 5'RACE PCR fragments generated with *zap*-specific primers were sequenced. In all the transcripts from the LG2080 maize plants, there was an insertion of additional sequence in the coding region, which made the ZAP protein longer by 11 amino acids (980 amino acids, molecular mass ~110.4 kDa). The Zn-finger I of ZAP2 was standard and read CKSCxxxHARC (Figure 1B; SEQ ID No 11). The sequence difference may be due either to differences between maize varieties, to the expression of two homologous genes, or to alternative splicing. In fact, maize may have at least two *zap* genes (Figure 2B), which encode a transcript of 3.4-3.5 kb (Figure 2D). The DNA gel blot experiment with a probe prepared from the *zap* cDNA showed that homologous genes were present in *Arabidopsis*.

Structurally ZAP was very similar to PARP from animals. It had a well conserved DNA-binding domain composed of two Zn-fingers (36% identity and 45% similarity to the DNA-binding domain of mouse PARP). Even higher homology was shown by comparing only the sequences of the Zn-fingers, Ala<sup>1</sup>-Phe<sup>162</sup> in the mouse enzyme (44% identity and 54% similarity), or a subdomain downstream from the nuclear

localization signal (NLS), Leu<sup>237</sup>-Ser<sup>360</sup> in mouse PARP (40% identity and 50% similarity). Whereas the bipartite nuclear localization signal characteristic of mammalian PARP could not be identified in ZAP, the sequence KRKK fitted a monopartite NLS (Figure 1B). The putative automodification domain was poorly conserved and was shorter in ZAP than in mouse PARP. The compilation of the homology of the catalytic domains between ZAP, NAP, APP and mouse PARP is shown in Figure 2. It should be noted that the NAD<sup>+</sup>-binding domain of ZAP was more similar to the mammalian enzyme (48% identity) than to that of APP and NAP (40% and 42% sequence identity, respectively), whereas APP and NAP were 68% identical and 76% similar in their catalytic domain.

**Example 2 Demonstration that non-conventional PARP protein has a DNA-dependent poly(ADP-ribose) polymerase activity.**

*APP is a DNA-dependent poly(ADP-ribose) polymerase*

A more detailed study of the APP protein (expressed in yeast) was performed to understand the activity of PARP-like proteins from the NAP class. The choice of yeast as the organism for the expression and enzymatic analysis of the *Arabidopsis* APP protein was made for a number of reasons. As an eukaryote, *Saccharomyces cerevisiae* is better suited for the expression of native proteins from other eukaryotic organisms, and unlike most other eukaryotic cells, it does not possess endogenous PARP activity (Lindahl *et al.*, 1995).

The full-length *app* cDNA was placed in pYeDP1/8-2 under the control of a galactose-inducible yeast promoter in the following way. the full-length *app* cDNA was excised from pC3 (Lepiniec *et al.*, 1995) as an *Xho*I-*Eco*RI fragment. The ends were filled in with the Klenow fragment of DNA polymerase I, and the fragment was subcloned into the *Sma*I site of the yeast expression vector pYeDP1/8-2 (Cullin and Pompon, 1988). The resulting expression vector pV8SPA (Figure 4A) was transformed into *S. cerevisiae* strain DY.

For APP expression in *E. coli*, the complete coding region of the *app* cDNA was PCR amplified with *Pfu* DNA polymerase (Stratagene), using the primers

5'-AGGATCCCATGGCGAACAAGCTCAAAGTGAC-3' (SEQ ID No 19) and 5'-AGGATCCTTAGTGCTTGTAGTTGAAT-3' (SEQ ID No 20), and subcloned as a *Bam*HI fragment into pET19b (Novagene, Madison, WI), resulting in pETSPA. The expression of the full-length APP in *E. coli* BL21 from pETSPA was very poor. To obtain better expression, pETSPA was digested with *Nco*I and *Nde*I or with *Sma*I, the ends were filled in by the Klenow fragment of DNA polymerase I, and the plasmids were then self-ligated. Of the resulting plasmids pETΔNdeSPA and pETΔSmaSPA, only pETΔNdeSPA gave satisfactory expression of the truncated APP polypeptide (Met<sup>310</sup> to His<sup>637</sup>) in *E. coli* BL21.

The expression of the APP in yeast was verified by Northern and Western blot analysis. (Fig 4) As the promoter in pV8SPA is inactive when cells are grown on glucose and derepressed on galactose-containing media, the expression was expected to be tightly regulated by the carbon source. However, Northern blot analysis of RNA and immunoblot analysis of proteins in DY(pV8SPA) as compared to the control DY strain containing the empty vector, showed that app mRNA and APP protein were expressed in yeast even when grown on glucose-containing media (Figure 4B, lane 2). The peculiarity of the expression observed on glucose-containing medium was that both app mRNA and APP protein were shorter than the ones detected after induction with galactose (compare lanes 2 and 4 in Figure 4B). The APP polypeptide with the higher molecular weight, (apparently a full-length protein) was only detected on galactose-containing medium, although such cells also expressed the truncated mRNA and protein. The most probable explanation for this finding is that when the DY(pV8SPA) strain is grown on glucose, there is a leaky expression from the expression cassette, with transcription beginning 200-300 bp downstream from the transcription start observed after galactose induction. This shorter mRNA probably does not code for the first methionine (Met<sup>1</sup>) of APP and, therefore, translation is initiated at Met<sup>72</sup>. This would explain the observed difference of -5 kDa (calculated difference being 7.5 kDa) in the molecular masses of the APP polypeptides from strains grown on glucose or on galactose. The possibility that the differences in molecular masses may be attributed to self-modification through poly(ADP-ribos)ylation was ruled out by growing strains in the presence of PARP

inhibitors, such as 3ABA and nicotinamide (Figure 4B, compare lanes 6 and 8 to lane 4).

To detect the synthesis of poly(ADP-ribose), total proteins were extracted from yeast strains grown under different conditions and incubated in the presence of radioactively labeled  $\text{NAD}^+$ . To prevent synthesis of poly(ADP-ribose) and possible automodification of the APP *in vivo*, strains were also grown in the presence of 3ABA, a reversible inhibitor of PARP, which was subsequently removed from the protein extracts during desalting. Figure 5 shows that poly(ADP-ribose) is synthesized by protein extracts of DY(pV8SPA) grown on galactose (Figure 5A, lanes 1 and 2), but not by a strain containing the empty vector (Figure 5A, lane 4). It can also be seen that *Arabidopsis* APP could synthesize polymers up to 40 residues in length (Figure 5A, lane 1) with the majority of the radioactivity being incorporated into 10-15-mer. This observation is consistent with the polymer sizes detected by other authors (Chen *et al.*, 1994). More radioactivity was incorporated into polymer when the yeast strain was grown with 3ABA than without (Figure 5A, lane 1 compared to lane 2); the reason might be that either the APP extracted from inhibited cultures was less automodified (it is believed that automodification inhibits the activity of PARP) or the labeled  $\text{NAD}^+$  was used by the enzyme from the uninhibited culture for the extension of existing polymer, resulting in a lower specific activity overall. Under the same reaction conditions poly(ADP-ribose) synthesized by human PARP, either in reaction buffer alone or in the presence of a yeast total protein extract from DY(pYeDP1/8-2) (Figure 5A, lanes 5 and 6, respectively), showed much longer chains, possibly up to 400-mer (de Murcia and Ménissier de Murcia, 1994).

The stimulation of enzymatic activity by nicked DNA is a well known property of PARP from animals (Alvarez-Gonzalez and Althaus, 1989). We therefore tested whether the activity of the APP protein was DNA dependent. After removal of yeast nucleic acids (DNA, RNA) and some basic proteins from the galactose-grown DY(pV8SPA) protein extract the synthesis of poly(ADP-ribose) was analyzed in the presence of increasing concentrations of sonicated salmon sperm DNA. As can be seen in Figure 5B, there was a direct correlation between the amount of DNA present in the reaction and the incorporation of  $^{32}\text{P}$ - $\text{NAD}^+$ . Scanning of the phosphor-images indicated that ~6-fold more radioactivity was incorporated into poly(ADP-ribose) in the reaction mixture

containing 40  $\mu\text{g ml}^{-1}$  of DNA than into that with 2  $\mu\text{g ml}^{-1}$  of DNA (Figure 5B, lanes 4 and 2, respectively). The synthesis of the polymer was sensitive to 3ABA in the reaction mix (Figure 5B, lane 5).

#### *APP is a nuclear protein*

In animal cells PARP activity is localized in the nucleus (Schreiber *et al.*, 1992). The intracellular localization, if nuclear, of APP could provide an important additional indication that APP is a *bona fide* plant PARP. To this end, the localization of the APP polypeptides in yeast cells was analyzed using anti-APP antisera. The APP polypeptide synthesized in yeast grown on galactose was found mainly in the nucleus. This localization was unaffected by the presence in the media of the PARP inhibitors.

In addition, we tested whether APP was constitutively active in yeast cells, as has been reported for the human PARP (Collinge and Althaus, 1994). Here, fixed yeast spheroplasts were incubated with monoclonal 10H antibody, which specifically recognizes poly(ADP-ribose) polymers (Kawamitsu *et al.*, 1984). A positive yellowish-green fluorescence signal with 10H antibody was localized in the nucleus and was observed only in DY(pV8SPA) cells grown on galactose. Positive staining was greatly reduced in cells grown in the presence of the PARP inhibitors, 3ABA and nicotinamide.

To identify the intracellular localization of APP in plant cells, a widely adopted approach in plant studies was used, *i.e.*, the examination of the subcellular location of a fusion protein formed between the protein in question and a reporter gene, once the protein fusion was produced in transgenic plants or transfected cells (Citovsky *et al.*, 1994; Sakamoto and Nagatani, 1996; Terzaghi *et al.*, 1997; von Arnim and Deng, 1994). An N-terminal translational fusion of GUS with the part of the APP polypeptide extending from the Met<sup>1</sup> to Pro<sup>407</sup> was made. The translational fusion of APP with bacterial GUS was constructed as follows. Plasmid pETSPA was cut with *Sma*I, treated with alkaline phosphatase, and ligated to a blunted *Nco*I-*Xba*I fragment from pGUS1 (Plant Genetic Systems N.V., Gent, Belgium). The ligation mix was transformed into *E. coli* XL-I and cells were plated onto LB medium supplemented with 0.1 mM isopropyl- $\beta$ -D-thiogalactopyranoside, 40  $\mu\text{g ml}^{-1}$



5-bromo-4-chloro-3-indolyl- $\beta$ -D-glucuronide, and 100  $\mu\text{g ml}^{-1}$  of ampicillin. In this way, pETSPAGUS was selected as blue colonies. The expression in *E. coli* of the ~110-kDa fusion protein was confirmed by *in situ* GUS activity gels (Lee *et al.*, 1995). The APP-GUS fusion was placed under the control of the 35S promoter of the CaMV (the Klenow-blunted *Bam*HI fragment from pETSPAGUS was subcloned into *Sma*I-digested pJD330; Gallie and Walbot, 1992) and the resulting expression cassette was subcloned as an *Xba*I fragment into the *Xba*I site of the pCGN1547 binary vector (McBride and Summerfelt, 1990) to give pGCNSPAGUS. The pGCNSPAGUS was finally introduced into *A. tumefaciens* C58C1Rif<sup>R</sup>(pGV2260) by the freezing-thawing transformation procedure.

Expression of the fusion protein was verified in *E. coli*. The chimeric cDNA under the control of the 35S CaMV promoter was stably integrated into the tobacco genome. Progeny from four independent transgenic tobacco plants were analyzed for the subcellular distribution of the GUS activity after *in situ* histochemical staining (Jefferson *et al.*, 1987). In 2-day-old seedlings GUS activity could be detected in cotyledons and in roots, but not in hypocotyls or root tips. Because of the transparency of root tissues, GUS staining was clearly localized in the nuclei of root hairs and epidermal cells. Additionally, some diffuse, non-localized staining of other root cells was seen, in particular along the vascular cylinders. This non-nuclear GUS staining was more pronounced in leaf tissues. Whereas young true leaves or cotyledons displayed intense blue staining of the nuclei, there was also some diffuse staining of the cytoplasm. In fully expanded leaves, however, GUS staining became homogenous and similar to the staining of control plants transformed with GUS under the control of the CaMV 35S promoter, in which GUS was expressed in the cytoplasm. Eventually, older leaves or cotyledons exhibited practically no histochemically detectable GUS activity, with the exception of the vascular bundles, where the GUS staining could not be confined to any particular cell compartment.

#### *Deficiency in DNA ligase I induces expression of the app gene*

PARP in animal cells is one of the most abundant nuclear proteins and its activity is regulated by allosteric changes in the protein upon binding to damaged DNA. We found that the *app* gene in *Arabidopsis* had a rather low level of expression,

suggesting that transcriptional activation of this gene might be essential for APP function *in vivo*. To test this hypothesis, the expression of the *app* gene was studied during *in vivo* genome destabilization caused by a DNA ligase I deficiency. A T-DNA insertion mutation, line SK1B2, in the *Arabidopsis* DNA ligase I gene was isolated previously (Babiychuk *et al.*, 1997). The mutation is lethal in the homozygous state, but the mutant allele shows normal transmission through the gametes. We therefore expected that cells homozygous for the mutation would die due to incomplete DNA synthesis during the S phase of the cell cycle, soon after the fertilization of the mutant embryo sac with mutant pollen.

An *app* promoter-GUS translational fusion, in which the coding region of GUS was fused in-frame with the first five amino acids of APP and 2 kb of *app* 5' flanking sequences was constructed (SEQ ID No 21). The gene encoding the fusion protein was transformed into *Arabidopsis*. After two back-crosses to a wild type, heterozygous plants transformed with *app* promoter-GUS were crossed with *Arabidopsis* line SK1B2. The inflorescences of the control plants and plants heterozygous for the ligase mutation were stained for the activity of GUS. The GUS staining pattern mostly detected in aging tissues probably reflects the expression of the *app* gene, although we have no firm evidence that all of the regulatory sequences were present in the constructs used. This pattern was the same both in the inflorescences of control plants, not carrying the mutant ligase gene and plants heterozygous for a mutation. Approximately one-fourth of the ovules in the mutant plants with the fusion protein are GUS positive. Closer microscopical examination showed that in the GUS-positive ovules only the gametophyte was stained. The only difference between the control plants and the mutant plant was a mutation in a DNA ligase gene. We therefore conclude that the *app* gene is induced because of either the accumulation of DNA breaks, or the death of the mutant embryo sacs fertilized with mutant pollen. GUS staining of embryo sacs was found to appear within 24 h after pollination, or therefore very soon after fertilization.

**Example 3. Construction of PCD modulating chimeric genes and introduction of the T-DNA vectors comprising such PCD modulating genes in an *Agrobacterium* strain.**

### 3.1. Construction of the p35S:(dsRNA-APP) and p35S:(dsRNA-ZAP) genes

Using standard recombinant DNA procedures, the following DNA regions are operably linked, as schematically outlined in Figure 6 (constructs 1 and 5):

For the p35S:(dsRNA-ZAP) chimeric gene

- a CaMV 35S promoter region (Odell *et al.*, 1985)
- a Cab22 leader region (Harpster *et al.*, 1988)
- a ZAP encoding DNA region (about complete) (the *Arabidopsis thaliana* homologue to SEQ ID No 10, isolated by hybridization)
- about 500 bp of the 5' end of the ZAP2 encoding DNA region in inverse orientation
- a CaMV35S 3' end region (Mogen *et al.*, 1990)

For the p35S:(dsRNA-APP) chimeric gene

- a CaMV 35S promoter region (Odell *et al.*, 1985)
- a Cab22 leader region (Harpster *et al.*, 1988)
- an APP encoding DNA region (about complete) (SEQ ID No 5)
- about 500 bp of the 5' end of the APP encoding DNA region in inverse orientation
- a CaMV35S 3' end region (Mogen *et al.*, 1990)

### 3.2. Construction of the pNOS:(dsRNA-APP) and pNOS:(dsRNA-ZAP) genes

Using standard recombinant DNA procedures, the following DNA regions are operably linked, as schematically outlined in Figure 6 (constructs 2 and 6):

For the pNOS:(dsRNA-ZAP) chimeric gene

- a NOS promoter region (Herrera-Estrella *et al.*, 1983)
- a Cab22 leader region (Harpster *et al.*, 1988)
- a ZAP encoding DNA region (about complete) (the *Arabidopsis thaliana* homologue to SEQ ID No 10, isolated by hybridization)
- about 500 bp of the 5' end of the ZAP2 encoding DNA region in inverse orientation
- a CaMV35S 3' end region (Mogen *et al.*, 1990)

For the pNOS:(dsRNA-APP) chimeric gene

- a NOS promoter region (Herrera-Estrella *et al.*, 1983)
- a Cab22 leader region (Harpster *et al.*, 1988)
- an APP encoding DNA region (about complete) (SEQ ID No 5)
- about 500 bp of the 5' end of the APP encoding DNA region in inverse orientation
- a CaMV35S 3' end region (Mogen *et al.*, 1990)

### 3.3. Construction of the pTA29:(dsRNA-APP) and pTA29:(dsRNA-ZAP) genes

Using standard recombinant DNA procedures, the following DNA regions are operably linked, as schematically outlined in Figure 6 (constructs 3 and 7):

For the pTA29:(dsRNA-ZAP) chimeric gene

- a TA29 promoter region (WO 89/10396)
- a Cab22 leader region (Harpster *et al.*, 1988)
- a ZAP encoding DNA region (about complete) (the *Arabidopsis thaliana* homologue to SEQ ID No 10, isolated by hybridization)
- about 500 bp of the 5' end of the ZAP2 encoding DNA region in inverse orientation
- a CaMV35S 3' end region (Mogen *et al.*, 1990)

For the pTA29:(dsRNA-APP) chimeric gene

- a TA29 promoter region (WO 89/10396)
- a Cab22 leader region (Harpster *et al.*, 1988)
- an APP encoding DNA region (about complete) (SEQ ID No 5)
- about 500 bp of the 5' end of the APP encoding DNA region in inverse orientation
- a CaMV35S 3' end region (Mogen *et al.*, 1990)

### 3.4. Construction of the pNTP303:(dsRNA-APP) and pNTP303:(dsRNA-ZAP) genes

Using standard recombinant DNA procedures, the following DNA regions are operably linked, as schematically outlined in Figure 6 (constructs 4 and 8):

For the pNTP303:(dsRNA-ZAP) chimeric gene

- a NTP303 promoter region (Wetering 1994)

- a Cab22 leader region (Harpster *et al.*, 1988)
- a ZAP encoding DNA region (about complete) (the *Arabidopsis thaliana* homologue to SEQ ID No 10, isolated by hybridization)
- about 500 bp of the 5' end of the ZAP2 encoding DNA region in inverse orientation
- a CaMV35S 3' end region (Mogen *et al.*, 1990)

For the pNTP303:(dsRNA-APP) chimeric gene

- a NTP303 promoter region (Wetering, 1994)
- a Cab22 leader region (Harpster *et al.*, 1988)
- an APP encoding DNA region (about complete) (SEQ ID No. 5)
- about 500 bp of the 5' end of the APP encoding DNA region in inverse orientation
- a CaMV35S 3' end region (Mogen *et al.*, 1990)

### 3.5 Construction of the chimeric marker genes

Using standard recombinant DNA procedures, the following DNA regions are operably linked, as schematically outlined in Figure 6:

For the *gat* marker gene

- an Act2 promoter region (An *et al.*, 1996)
- a aminoglycoside 6'-acetyltransferase encoding DNA (WO 94/26913)
- a 3' end region of a nopaline synthase gene (Depicker *et al.*, 1982)

For the *bar* marker gene

- an Act2 promoter region (An *et al.*, 1996)
- a phosphinotricin acetyltransferase encoding DNA (US 5,646,024)
- a 3' end region of a nopaline synthase gene (Depicker *et al.*, 1982)

### 3.6. Construction of the T-DNA vectors comprising the PCD modulating chimeric genes

Using appropriate restriction enzymes, the chimeric PCD modulating genes described under 3.1 to 3.5 are excised and introduced in the polylinker between the T-DNA borders of a T-DNA vector derived from pGSV5 ( WO 97/13865) together

with either the *gat* marker gene or the *bar* marker gene. The resulting T-DNA vectors are schematically represented in Figure 6.

### 3.7. Introduction of the T-DNA vectors in *Agrobacterium*

The T-DNA vectors are introduced in *Agrobacterium tumefaciens* C58C1Rif(pGV4000) by electroporation as described by Walkerpeach and Velten (1995) and transformants are selected using spectinomycin and streptomycin.

#### **Example 4. *Agrobacterium*-mediated transformation of *Arabidopsis thaliana* with the T-DNA vectors of Example 3.**

The *Agrobacterium* strains are used to transform *Arabidopsis thaliana* var. C24 applying the root transformation method as described by Valvekens et al. (1992). The explants are coinfectd with the *Agrobacteria* strains containing the dsRNA-APP respectively the dsRNA-ZAP constructs. The dsRNA-APP constructs are used in combination with the *pact:bar* gene. The dsRNA-ZAP constructs are used in combination with the *pact:gat* gene. Transformants are selected for phosphinothricin resistance. The regenerated rooted transgenic lines are tested for the presence of the other T-DNA by screening for kanamycin resistance. Transgenic lines containing both T-DNA's are transfered to the greenhouse. The phenotype of the T0-transgenic lines is scored and the T1-generations are studied further in more detail.

#### **Example 5. *Agrobacterium*-mediated transformation of *Brassica napus* with the T-DNA vectors of Example 3.**

The *Agrobacterium* strains are used to transform the *Brassica napus* var. N90-740 applying the hypocotyl transformation method essentially as described by De Block et al. (1989), except for the following modifications:

- hypocotyl explants are precultured for 1 day on A2 medium [MS, 0.5 g/l Mes (pH5.7), 1.2% glucose, 0.5% agarose, 1 mg/l 2,4-D, 0.25 mg/l naphthalene acetic acid (NAA) and 1 mg/l 6-benzylaminopurine (BAP)].

- infection medium A3 is MS, 0.5 g/l Mes (pH5.7), 1.2% glucose, 0.1 mg/l NAA, 0.75 mg/l BAP and 0.01 mg/l gibberellin acid (GA3).
  - selection medium A5G is MS, 0.5 g/l Mes (pH5.7), 1.2% glucose, 40 mg/l adenine.SO<sub>4</sub>, 0.5 g/l polyvinylpyrrolidone (PVP), 0.5% agarose, 0.1 mg/l NAA, 0.75 mg/l BAP, 0.01 mg/l GA3, 250 mg/l carbenicillin, 250 mg/l triacillin, 5 mg/l AgNO<sub>3</sub> for three weeks. After this period selection is continued on A5J medium (similar a A5G but with 3% sucrose)
  - regeneration medium A6 is MS, 0.5 g/l Mes (pH5.7), 2% sucrose, 40 mg/l adenine.SO<sub>4</sub>, 0.5 g/l PVP, 0.5% agarose, 0.0025mg/l BAP and 250 mg/l triacillin.
  - healthy shoots are transferred to rooting medium which was A9: half concentrated MS, 1,5% sucrose (pH5.8), 100 mg/l triacillin, 0.6 % agar in 1 liter vessels.
- MS stands for Murashige and Skoog medium (Murashige and Skoog, 1962)

For introducing both the dsRNA-APP and the dsRNA-ZAP T-DNA constructs into a same plant cell the co-transformation method is applied, essentially as described by De Block and Debrouwer (1991). Transformed plant lines are selected on phosphinothricin containing medium after which the presence of the second T-DNA is screened by testing the regenerated rooted shoots for kanamycin resistance. In the co-transformation experiments, the dsRNA-APP constructs are used in combination with the *pact:bar* gene. The dsRNA-ZAP constructs are used in combination with the *pact:gat* gene. Transgenic lines containing both T-DNA's are transferred to the greenhouse. The phenotype of the T0-transgenic lines is scored and the T1-generations are studied further in more detail.

## **Example 6. In vitro assay to test vigor of plant lines**

### **6.1. Fitness assay for *Brassica napus***

#### ***Media and reaction buffers***

##### **Sowing medium:**

Half concentrated Murashige and Skoog salts  
 2% sucrose  
 pH 5.8

0.6% agar

Callus inducing medium: A2S

MS medium, 0.5g/l Mes (pH 5.8), 3% sucrose, 40mg/l adenine-SO<sub>4</sub>, 0.5% agarose, 1mg/l 2,4-D, 0.25mg/l NAA, 1mg/l BAP

Incubation medium:

25mM K-phosphate buffer pH5.8

2% sucrose

1 drop Tween20 for 25ml medium

Reaction buffer:

50mM K-phosphate buffer pH7.4

10mM 2,3,5-triphenyltetrazoliumchloride (TTC) (= 3.35mg/ml)

1 drop Tween20 for 25ml buffer

### *Sterilization of seeds and growing of the seedlings*

Seeds are soaked in 70% ethanol for 2 min, then surface-sterilized for 15 min in a sodium hypochlorite solution (with about 6% active chlorine) containing 0.1% Tween20. Finally, the seeds are rinsed with 1l of sterile distilled water. Put 7 seeds/1l vessel (Weck) containing about 75ml of sowing medium. The seeds are germinated at 23°C and 30  $\mu\text{Einstein/s}^{-1}\text{m}^{-2}$  with a daylength of 16h.

The line N90-740 is always included for standardization between experiments.

### *Preculture of the hypocotyl explants*

- 12-14 days after sowing, the hypocotyls are cut in about 7mm segments.  
25 hypocotyls/Optilux Petridisch (Falcon S1005)
- The hypocotyl explants are cultured for 4 days on medium A2S at 23-25°C (at 30 $\mu\text{Einstein/s}^{-1}\text{m}^{-2}$ ).  
□ P.S.: about 150-300 hypocotyl explants/line are needed to carry out the assay
- Transfer the hypocotyl explants to Optilux Petridishes (Falcon S1005) containing 30ml of incubation medium.
- Incubate for about 20hours at 24°C in the dark.

### *TTC-assay*

- Transfer 150 hypocotyl explants to a 50ml Falcon tube.
- Wash with reaction buffer (without TTC).



- Add 25ml-30ml of reaction buffer/tube.
- tube 1 ☐ no TTC added
  - \* for measuring background absorption
  - \* one line/experiment is sufficient
- tube 2 ☐ +10mM TTC
  - (explants have to be submerged, but do not vacuum infiltrate!)
- turn tubes upside down
- Incubate for about 1 hour in the dark at 26°C (no end reaction!)
- Wash hypocotyls with deionized water
- Remove water
- Freeze at -70°C for 30min.
- Thaw at room°t (in the dark)
- Add 50ml ethanol (technical)
- Extract reduced TTC-H by shaking for 1 hour
- Measure absorptions of extracts at 485nm

P.S.: reduced TTC-H is not stable ☐ keep in the dark and measure O.D.<sub>485</sub> as soon as possible

$$\text{O.D.}_{485} (\text{TTC-H}) = (\text{O.D.}_{485} + \text{TTC}) - (\text{O.D.}_{485} - \text{TTC})$$

- Comparison of the TTC-reducing capacities between samples of different independent experiments can be done by setting the TTC-reducing capacity of N90-740 in the different experiment at 100%.
- Lines with a high TTC-reducing capacity are vigorous, while lines with a low TTC-reducing capacity are weak.

## 6.2. Fitness assay *Arabidopsis*

### *Media and reaction buffers*

Plant medium: Half concentrated Murashige and Skoog salts

1.5% sucrose

pH 5.8

0.6% agar

→ autoclave 15min.

add filter sterilized -100mg/l myo-inositol

- 0.5mg/l pyridoxine
- 0.5mg/l nicotinic acid
- 1mg/l thiamine

Incubation medium: 10mM K-phosphate buffer pH5.8

2% sucrose

1 drop Tween20 for 25ml medium

Reaction buffer: 50mM K-phosphate buffer pH7.4

10mM 2,3,5-triphenyltetrazoliumchloride (TTC) (= 3.35mg/ml)

1 drop Tween20 for 25ml buffer

### *Arabidopsis plants*

#### - Sterilization of *Arabidopsis* seeds

2min. 70% ethanol

10 min. bleach (6% active chlorine) + 1drop Tween 20 for 20ml solution

wash 5 times with sterile water

P.S.: sterilization is done in 2ml eppendorf tubes

*Arabidopsis* seeds sink to the bottom of the tube, allowing removal of the liquids by means of a 1ml pipetman

#### - Growing of *Arabidopsis* plants

Seeds are sown in 'Intergrid Tissue Culture disks of Falcon' (nr. 3025) containing  $\pm 100\text{ml}$  of plant medium: 1 seed/grid.

Plants are grown at 23°C

$40\mu\text{Einstein s}^{-1}\text{m}^{-2}$

16hours light - 8hours dark

for about 3 weeks (plants start to form flower buds)

→ P.S.: \*about 90-110 plants/line are needed to carry out the assay

\* include control line (C24; Columbia; ...) for calibration

### *Pre-incubation*

#### - Harvest *Arabidopsis* shoots by cutting of roots (by means of scissors)

Put each shoot immediately in incubation medium (shoots have to be submerged, but do not vacuum infiltrate)

Incubation medium:  $\pm 150$ ml in 'Intergrid Tissue Culture disks of Falcon' (nr. 3025)

- a) incubation medium: for quantification of background absorption (see TTC-assay)
- b) incubation medium
- c) incubation medium + 2mM niacinamide

30 - 35 shoots/petridish (but same amount of shoots for all lines and for each condition)

- Incubate at 24°C in the dark for  $\pm 20$ hours

#### TTC-assay

- Transfer shoots to 50ml Falcon tubes
- Wash with reaction buffer (without TTC)
- Add 30-35ml of reaction buffer/tube
  - a) no TTC added (for measuring background absorption)
  - b and c) +10mM TTC
- (Shoots have to be submerged, but do not vacuum infiltrate!)
- Incubate for about 2hours in the dark at 26°C (**no** end reaction!)
- Wash shoots with deionized water
- Remove water
- Freeze at -70°C for 30min.
- Thaw at room°t (in the dark)
- Add 50ml ethanol (technical)
- Extract reduced TTC-H by shaking for 1hour
- Measure absorptions of extracts at 485nm

P.S.: reduced TTC-H is not stable → keep in the dark and measure O.D.<sub>485</sub> as soon as possible

- Compare reducing profiles of tested lines *versus* control line (for population of 30 to 35 plants)

$$\text{O.D.}_{485}(\text{TTC-H}) = (\text{O.D.}_{485} + \text{TTC}) - (\text{O.D.}_{485} - \text{TTC})$$

- Comparison of the TTC-reducing capacities between samples of different independent experiments can be done by setting the TTC-reducing capacity of control line (C24; Columbia; ...) in the different experiments at 100%.

- Lines with a high TTC-reducing capacity are vigorous, while lines with a low TTC-reducing capacity are weak.
- If the addition of niacinamide to the incubation medium results in a higher TTC-reducing capacity indicates to a lower fitness (as shown for C24 and Columbia).

**Example 7. Phenotypic analyses of the transgenic lines containing both dsRNA-APP and dsRNA-ZAP constructs.**

The flower phenotype and pollen viability (Alexander staining (Alexander, 1969) and germination assay) of the T0-lines containing dsRNA-APP and dsRNA-ZAP under the control of tapetum or pollen specific promoters were scored. For *Arabidopsis*, the T1-generation is obtained by selfing or if the plants are male sterile by backcrossing using pollen of non-transformed wild type plants. For *Brassica napus*, the T1-generation is always obtained by backcrossing using pollen of non-transformed plants.

T1-seed is germinated on kanamycin containing medium after which the resistant plants are scored by means of the ammonium-multiwell assay for phosphinothricine resistance (De Block et al., 1995). One half of the plants that contains both T-DNA's is transferred to the greenhouse to score the male fertility of the plants, while the other half is used to quantify the vigor of the plants by means of the fitness assay.

For plants comprising combinations (APP/ZAP) of PCD modulating genes under control of 35S or NOS promoter, a high vigor is observed in a number of the transgenic lines.

For plants comprising combinations (APP/ZAP) of PCD modulating genes under control of TA29 male sterility is observed in a number of the transgenic lines.

For plants comprising combinations (APP/ZAP) of PCD modulating genes under control of NTP303 sterile pollen is observed in a number the transgenic lines.

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